UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS VETERINARIA

Departamento de Producción Animal

TESIS DOCTORAL

La coneja como modelo animal para el estudio del crecimiento intrauterino retardado inducido por restricción alimentaria gestacional

The rabbit as a model for studying intrauterine growth restriction induced by maternal food restriction

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Jorge López Tello

Directores

María Arias Álvarez
Pilar García Rebollar
Antonio González de Bulnes

Madrid, 2017

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Memoria para optar al grado de Doctor presentada por:
Jorge López Tello

Bajo la dirección de los Doctores:
María Arias Álvarez
Pilar García Rebollar
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Madrid, 2016

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Hacen constar que D. Jorge López Tello, Licenciado en Veterinaria, ha realizado bajo su dirección y supervisión el trabajo titulado: "LA CONEJA COMO MODELO ANIMAL PARA EL ESTUDIO DEL CRECIMIENTO INTRAUTERINO RETARDADO INDUCIDO POR RESTRICCIÓN ALIMENTARIA GESTACIONAL".

Se considera que la presente Memoria reúne a nuestro juicio la debida calidad y las condiciones de originalidad y rigor metodológico necesarios para su presentación y defensa ante el Tribunal correspondiente para optar al título de Doctor.

En Madrid, Noviembre de 2016

Fdo: M. Arias Álvarez Fdo: P. García Rebollar Fdo: A. González de Bulnes López

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This thesis has been proposed for the European doctorate mention by virtue of the following European research stay:

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“Luck is not a factor, hope is not a strategy and fear is not an option.
Make your own luck by rigorous application of a robust process!”

James Cameron

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BLR	Brain weight respect liver weight ratio
BPD	Biparietal diameter
c-GMP	Cyclic guanosine monophosphate
CPR	Cerebroplacental ratio
CT	Cytotrophoblast
D	Decidua
Db	Decidua basalis
DOHaD	Developmental Origins of Health and Disease
DV	Ductus venosus
eNOS	Endothelial nitric oxide synthases
FAO	Food and Agriculture Organization of the United Nations
GLUTs	Glucose transporters
HDL-c	High-density lipoproteins
Igf2	Insulin like growth factor II
iNOS	Inducible NO synthases
IP	Pulsatility index
IR	Resistance index
IUGR	Intrauterine growth restriction
IYS	Inverted YS
Jz	Junctional zone
LBW	Low body weight
LDL-c	Low-density lipoproteins
LGA	Large for Gestational Age
L-NAME	L-nitro-arginine methyl ester
Lz	Labyrinth zone
MCA	Middle cerebral artery
MFR	Maternal food restriction
NO	Nitric oxide
NOS	Nitric oxide synthases
NP	Non-pregnant state
NS	Nervous system
p.c.	*Post coitum*
p.p.	*Post partum*
PDE-5	Phosphodiesterase type-5
SC	Sildenafil Citrate
SGA	Small for Gestational Age
ST	Syncytiotrophoblasts

T	Trophectoderm
TB	Trophoblast
TBs	Trophoblast cells
UA	Uterine artery
UCA	Umbilical cord arteries
VYS	Visceral yolk sac
WHO	World Health Organization
YS	Yolk sac

Resumen

Resumen

El crecimiento del feto depende, en gran medida, de un óptimo estado nutricional materno y un correcto desarrollo placentario, siendo estos dos factores agentes limitantes de su potencial genético. Una pauta alimentaria incorrecta o deficiente durante la gestación puede predisponer al desarrollo de ciertas complicaciones médicas a corto y/o largo plazo, tanto en la madre como en su descendencia.

Entre las complicaciones fetales que aparecen con más frecuencia destaca el llamado Crecimiento Intrauterino Retardado (CIR), que se basa en una reducción del desarrollo fetal dando lugar a individuos con bajo peso y con alteraciones funcionales y/o en el tamaño de sus órganos al nacimiento. Esta desproporción suele estar asociada a cambios en determinados órganos, como el cerebro, hígado o páncreas, que pueden predisponerles a desarrollar ciertas enfermedades en edad adulta. La etiología del CIR es multifactorial, si bien la nutrición materna juega un papel fundamental. Pautas alimentarias incorrectas durante la gestación, tales como la alteración en los componentes de la dieta o la restricción de determinados nutrientes pueden afectar la funcionalidad de la placenta y, por tanto, la cantidad de alimento y oxígeno que llegan al feto en desarrollo. En el caso de la restricción alimentaria, estos posibles cambios dependerán, en gran medida, de la intensidad, del periodo de exposición y del tipo de restricción.

Las limitaciones tanto éticas como prácticas que conlleva la realización de estudios intervencionales en medicina humana determinan el empleo de modelos animales. De hecho, gran parte del avance obtenido en el conocimiento sobre las complicaciones gestacionales y sus posibles consecuencias en el desarrollo fetal y placentario se ha obtenido a través del uso de animales de experimentación; principalmente, de roedores y ovinos. Sin embargo, existen otros modelos animales como la coneja, que son válidos para el estudio de la insuficiencia placentaria y/o CIR, ya que presentan, entre otras ventajas, una placentación de tipo hemocorial y un desarrollo de determinados órganos fetales, como el cerebro, que presenta similitudes con la especie humana.

Además, el conejo se explota con fines productivos en los países mediterráneos, por lo que tiene una doble funcionalidad, como modelo biomédico y animal de producción. Por consiguiente, los

resultados obtenidos en pruebas experimentales realizadas en la coneja, como aquellas basadas en la reducción del aporte nutricional a la madre gestante, además de permitir estudiar las consecuencias sobre la función placentaria y el desarrollo fetal, permiten avanzar en el conocimiento sobre los posibles efectos que tiene la aplicación de dichas pautas en las granjas cunícolas. En este sentido, la reducción del coste en alimentación puede significar un beneficio económico muy importante para el ganadero debido a que la alimentación supera el 60% de los costes de producción. Por ello, la restricción alimentaria o ajustar el consumo a las necesidades fisiológicas de la reproductora, podría ser una estrategia de manejo a estudiar, ya que implicaría un ahorro económico para el cunicultor. Este manejo podría aplicarse especialmente en aquellos casos en los que la hembra se encuentra en las primeras etapas de la gestación y sus necesidades metabólicas o energéticas todavía no son máximas.

Así pues, el objetivo general de la presente Tesis Doctoral ha sido estudiar, en la coneja, los posibles efectos que genera la aplicación de pautas de restricción alimentaria gestacionales (RAG) del 50% en distintos momentos de la gestación a nivel materno, fetoplacentario y neonatal. Específicamente se han evaluado: 1) los cambios hemodinámicos sufridos a nivel placentario y fetal, así como la morfometría de la descendencia antes y después del nacimiento, 2) los efectos de un tratamiento vasodilatador a base de Citrato de Sildenafilo y 3) las consecuencias en el perfil metabólico de la madre, en la estructura histológica de la placenta y en la biometría fetal dependiendo de la fase de la gestación en la que la RAG es aplicada.

En el primer experimento de la Tesis Doctoral se evaluó el efecto que tiene la aplicación de la RAG al 50% una vez el embrión ya está implantado y comienza la formación de la placenta (día 9) hasta el final de la gestación (día 31). En este trabajo se evaluaron los cambios hemodinámicos placentarios (arteria umbilical; UCA) y fetales (arteria cerebral media; MCA), así como la morfometría fetal al final del segundo tercio de gestación. Posteriormente, en el momento del parto, se valoraron las consecuencias de la RAG sobre el peso corporal materno y el desarrollo de su descendencia, así como las tasas de mortalidad neonatal. La viabilidad y fiabilidad de la técnica ecográfica y del examen Doppler como métodos no invasivos para la detección precoz de los cambios en los patrones de crecimiento y hemodinámica fetales en esta especie quedaron confirmadas. Los fetos expuestos a RAG evidenciaron menores velocidades diastólicas y mayores velocidades sistólicas a nivel de la UCA que los controles, aumentando la velocidad media en esta arteria, que tendió a presentar mayores índices de pulsatilidad y de

resistencia. Estas alteraciones fueron debidas posiblemente a un deterioro en la funcionalidad placentaria por isquemia, que secundariamente generó también reducciones en la biometría fetal. No obstante, la RAG no afectó al peso corporal de la hembra y tampoco se observó una mayor mortalidad neonatal respecto al grupo alimentado *ad libitum,* a pesar del reducido tamaño de los recién nacidos.

En el segundo experimento de esta Tesis Doctoral se abundó en el estudio previo utilizándose el mismo periodo e intensidad de RAG, pero añadiéndose un grupo experimental tratado con Citrato de Sildenafilo (SC) en el último tercio de gestación. Los datos de las conejas sometidas a RAG obtenidos a día 26-28 de gestación reforzaron los resultados del desarrollo placentario y fetal determinados previamente. A nivel histológico las placentas del grupo restringido mostraron una decidua más delgada y un incremento del tejido conjuntivo fibroso desde la zona de unión hacia el laberinto que reemplazaba la superficie vascularizada en esa área. El tamaño y el peso de los fetos disminuyeron significativamente. La administración de SC generó cambios en el tamaño de la placenta y mejoró la vascularización, principalmente en la zona del laberinto y de la decidua, pudiendo contrarrestar parcialmente los efectos adversos de dicha RAG, compensando la biometría de los fetos y de los neonatos, aunque no mejoró significativamente el peso del individuo. Sin embargo, algunos datos referentes a la funcionalidad cerebral y hepática del feto sugirieron un posible aumento en el flujo cerebral del feto y una vasoconstricción compensatoria cuyas consecuencias en la descendencia a corto y largo plazo necesitan de más estudios.

En el tercer experimento, se compararon los efectos de la RAG al 50% aplicada en el periodo preimplantacional (día 0 a día 7 de gestación) y durante toda la gestación (día 0 a día 31 de gestación) frente a una alimentación *ad libitum*, complementando así el estudio anterior. En ambos periodos, la RAG no indujo cambios macroscópicos en el peso de la coneja, ni metabólicos a nivel glucémico ni en la leptinemia. Sin embargo, el metabolismo lipídico se vió alterado en las fases tempranas-medias de gestación, cuando se está formando la placenta, pero las necesidades nutricionales del futuro feto todavía son bajas. Estas diferencias desaparecieron en el último tercio de gestación, cuando la madre presenta un metabolismo principalmente catabólico y el feto necesita un mayor aporte de nutrientes para poder llevar a cabo su crecimiento exponencial. Además, la RAG indujo cambios histopatológicos en la placenta, que reflejaron un incremento de las zonas de fibrosis de la decidua, una reducción de la vascularización y del número de trofoblastos en el laberinto, así como un incremento

significativo del porcentaje de apoptosis y de expresión de caspasa-3 en las tres partes de la placenta. Estos hallazgos fueron más acusados en el grupo de hembras restringidas durante toda la gestación. A consecuencia de ello, se pudo observar que el tamaño fetal en el día 28 de gestación se vio afectado en los dos grupos restringidos, aunque el peso del feto solo disminuyó significativamente en el grupo en que la RAG fue mantenida durante todo el periodo de gestación. Además, los fetos presentaron alteraciones en la proporción de sus órganos, con una significativa reducción del peso del hígado.

En conclusión, el análisis conjunto de los resultados obtenidos en los diferentes experimentos indica que: 1) la RAG indujo cambios en la hemodinámica de la UCA y de la MCA. Todo ello generó cambios fenotípicos en el desarrollo fetal como reducción de peso, longitud o menores diámetros torácicos o biparietales, afectando también al desarrollo de ciertos órganos como el cerebro o el hígado, aunque el número de fetos desarrollados y neonatos no se modificó en ninguno de los casos, 2) estos resultados pueden ser parcialmente contrarrestados con la administración de SC aunque los posibles efectos vasodilatadores a nivel de la MCA deben ser estudiados y, por último, 3) las pautas de RAG estudiadas no produjeron cambios morfológicos en la madre, ni en sus concentraciones plasmáticas de leptina ni en su metabolismo glucídico. Sin embargo, alteraron su metabolismo lipídico en los periodos tempranos y medios de gestación, generando cambios en el fenotipo macroscópico y microscópico de la placenta de distinta manera e intensidad según el periodo en el cual la RAG fue aplicada.

Summary

Summary

Fetal growth depends on an adequate maternal nutritional balance and a correct placental development. Imbalanced diets during gestation may predispose the mother and her offspring to suffering from pregnancy complications and abnormal fetal development, with long-term consequences for their health.

Intrauterine Growth Restriction (IUGR), defined as the failure of a fetus to reach its genetic potential size, can be associated to multiple factors, such as placental insufficiency or maternal pathological diseases (*e.g.* undernutrition). In IUGR, the impairment in placental function reduces the oxygen and nutrient supply to the fetus, resulting in fetuses with low body weight at birth and possible disproportionate body growth. Such alteration may be associated with changes in the growth pattern of certain organs, such as the brain or the liver. However, the severity of the effects of undernutrition during gestation will depend on the time of exposure, the degree or intensity of the restriction and the type of food deprivation.

Despite the multiple differences in placentation and fetal development among eutherian mammals, experimental animals such as rodents or sheep have been useful for the study of IUGR and placental insufficiency. However, in the last years, there is a tendency to complete the results obtained from the aforementioned animal models with other eutherian mammals. In this sense, the employment of animals with a double functionality, biomedical modeling and livestock production, could reduce overlapping research and be a suitable alternative to the frequent employment of rodents. In this sense, the rabbit, which shares some physiological characteristics with the human (hemochorial placentation and accelerated fetal brain development) could be a suitable alternative.

In the Mediterranean area, the rabbit is a well-established livestock animal (mainly for meat production). However, in recent years, the costs of feeding animals have undergone a significant increase, which can be more than 60% of the global costs of production. Thereby, many farmers are forced to establish food restriction protocols in their farms in order to reduce productive costs. This management may be beneficial when applied in the early stages of the pregnancy, as maternal metabolic necessities and fetal requirements for growth are low. However, the

consequences of such restriction in placental and offspring outcome in rabbits are not well established.

Thereby, the objective of this thesis is to study, in the rabbit, the effects that a 50% maternal food restriction (MFR) application could generate in maternal, fetoplacental and neonatal statuses when this nutritional regimen is conducted in different gestational periods. Specifically, we have evaluated: 1) hemodynamic changes of the conceptus and morphometric parameters of the offspring in utero and at birth, 2) the effects of a vasodilator molecule based on the administration of Sildenafil Citrate (SC) and 3) the consequences that 50% MFR application has on maternal metabolism, placental structure and biometric measurements of the fetus.

The objective of the first work conducted in this thesis was to investigate the impact of 50% MFR carried out from the post-implantational period (day 9 of pregnancy) to the end of gestation (day 31 of pregnancy). In this work, we have evaluated conceptus hemodymanic changes (umbical cord arteries, UCA, and middle cerebral artery, MCA) by Doppler ultrasonography and fetal morphometry by echography at the end of the second third of gestation. At birth, we evaluated the effects of 50% MFR in the weight of the dams and the offspring outcome, in terms of biometry and mortality rate. The results of this trial reinforced the use of non-invasive techniques (ecography and Doppler ultrasonography) to detect early fetal growth disruption and changes in blood flow. Fetuses exposed to 50% MFR demonstrated lower end diastolic and higher systolic velocities at the UCA that generated changes in the mean time velocity and a tendency to higher pulsatility and resistance indexes respect well-nourished fetuses. Such alterations may be associated to a possible placental ischemia, also generated reductions in fetal biometry. Nevertheless, 50% MFR did not result in maternal body weight loss or higher neonatal death despite the reduced phenotype of the offspring.

The second experiment of the thesis further investigated the findings of the previous study. In this sense, we employed the same level of MFR and the same period of exposure. Thus, we added an extra group that was treated with SC in the last third of gestation. The results of this trial, obtained on day 26-28 of pregnancy, reinforced the previous study, specifically, the reduced phenotype observed in the offspring and the alterations in placental level. Histologically, we observed that undernourished placentas changed at the decidua, showing a thinned section and increased amounts of poorly cellular fibrous connective tissue that extended multi-focally into the

labyrinth and surrounds, and replaced vascular channels with the collapse of the adjacent labyrinth structure. Fetal weight was significantly reduced. The treatment with SC improved placental development by increasing vascularity and vessel hypertrophy in the decidua and labyrinth. Nevertheless, although SC improved certain biometric parameters of the offspring, it could not improve the weight of the offspring. Thus, the administration of SC could have had resulted in blood flow alterations at the cerebral level and alterations in the hepatic function of the fetus that warrant further investigations.

Finally, in the third experiment of this thesis, we decided to explore other time windows of the rabbit gestation. We sought to determine the consequences of the 50% MFR during the preimplantational period (from day 0 to day 7 of the pregnancy) or throughout gestation (day 0 onwards). In both periods, 50% MFR did not result in maternal body weight loss or in alterations in leptin or carbohydrates metabolites. However, 50% MFR resulted in maternal lipid alterations in early to mid stages of the pregnancy, when placental formation begins but fetal necessities for growth are minimal. These alterations in lipid metabolities disappeared in late gestation, when maternal metabolism is catabolic and the fetus requires more nutrients to support its growth. The application of 50% MFR induced histopathological changes at the decidua (*e.g.* fibrosis) or reduced vascularization and number of trophoblast at the labyrinth zone. Furthermore, higher apoptotic levels and caspase-3 expression were found in undernourished placentas, in a more severe grade in placentas exposed to MFR during the whole gestation. Consequently, fetal growth, determined on day 28 of gestation, was affected in both restricted groups, although fetal weight was only reduced in the most challenged group. Furthermore, fetuses exposed to MFR evidenced alterations in the proportion of their organs, with a significant reduction of liver growth in both restricted groups.

In conclusion, the data obtained from the different trials conducted over the period of this thesis suggest that: 1) MFR induced changes in the hemodynamic of the UCA and MCA. These changes altered the phenotype of the offspring, such as a reduced weight or shorter crown-rump length or lower thoracic and biparietal diameters. Thus, the development of certain organs, such as the brain or the liver was affected. However, the number of fetuses/neonates per female was not affected by MFR; 2) These results can be partially counteracted with the administration of SC in gestation, although the changes observed at the MCA require of further studies; lastly, 3) the protocols of MFR applied did not alter maternal body weight, leptin or glucidic metabolites.

However, these managements can affect lipid metabolites in early to mid stages of the pregnancy and alter the macro- and microscopic phenotype of the placenta in different manners according to the period of exposure to MFR.

Chapter 1: Introduction

Overview

Placentation evolved as a reproductive strategy to ensure an adequate environment to the developing fetus. This transient organ a part from anchoring the fetus to the uterine wall and preventing immunological rejection of the fetus by the maternal immune system, enables nutrient and gases exchange. However, the success of this fascinating mammalian strategy relies on an adequate maternal nutritional status and metabolism. Thereby, imbalances in the allocation of resources due to poor maternal food intake or impairment in placental formation and/or function can lead to pregnancy complications and abnormal fetal development.

Intrauterine growth restriction (IUGR) is defined as the failure of a fetus to reach its genetic potential size. This impairment in fetal growth can be associated to different etiologies, such as genetics, infections, malnutrition or placental insufficiency. These last two conditions are highly associated, in fact, the reduced maternal intake can impair placental function, which reduces utero-placental blood flow and consequently lowers oxygen and nutrient uptake by the fetus. As a result, the fetus may develop innate mechanisms of blood shunting to safeguard the growth of key organs, like the brain, even at the expense of the growth of other tissues (*e.g.* liver or skeletal muscle).

Overall, the availability of a reliable animal model to study fetoplacental characteristics in IUGR pregnancies would be highly beneficial in order to develop diagnostic, preventive and therapeutic strategies in humans and animals. In the last years, the rabbit has emerged as a valuable model to investigate IUGR. The rabbit placenta develops as a discoid hemochorial placentation such as that of the human and rodents, and hemodynamic changes occurring during pregnancy are comparable with the human, with an important increase in maternal blood pressure throughout gestation. Thus, rabbit fetuses have an accelerated brain growth, being more similar to humans respect rodents. In addition, this model allows different approaches to induce placental insufficiency and IUGR (*e.g.* surgical procedures, pharmacological treatments or dietary protocols).

Therefore, to provide a framework for the work conducted, the first two themes of this introduction will broadly review the different gestational phases and the important of an adequate maternal

nutrition and metabolism in gestation. The third theme will provide an overview of the effects that malnutrition can generate in conceptus development and growth (*e.g.* IUGR). The fourth part of the literature review will be focus on the current biomedical models employed to study IUGR. Finally, the fifth argument of the introduction will be dedicated to the rabbit as a model. In this section, the gestational phases, placentation and fetal growth of the rabbit will be detailed. Moreover, we will describe the methods for inducing placental insufficiency and IUGR in this model and finally we will introduce the rabbit as a livestock animal.

1. Pregnancy & placentation

The pregnancy, also known as gravidity or gestation, is the carrying of an embryo or fetus inside the viviparous female. The time interval of a gestation is called the gestation period and, in mammals, begins when the zygote is formed and ends when the fetus leaves the uterus. The different processes that take place during the eutherian gestation such as preimplantation embryo development, implantation, placental formation and fetal development occur with great diversity among species. These differences are mainly based on the morphology and structure of the uterus, developmental stage of the embryo at the time of implantation and on endocrine and molecular interactions between the uterine and the embryonic tissues (Chavatte & Guillomot, 2007).

1.1. Preimplantational phase

In general terms, the preimplantation embryo development comprises the initial stages of mammalian development, before the embryo implants into the mother's uterus. This period begins after fecundation, when the zygote (Figure 1) multiplies its cells by several mitotic divisions. The result of these divisions is an increased number of embryonic cells or blastomeres. The embryo passes through different preimplantational embryonic stages, from 2 to 8 and 16 cells, and then it is compacted reaching the morula stage (Figure 1). When blastomeres migrate to form a central cavity, known as blastocele, the embryo enters into blastocyst stage (Figure 1) (Mescher, 2013). The way these processes occur differ among species. From the moment that the blastocele becomes the yolk sac (YS) and the zona pellucida is lost, the embryonic development enters into a new phase in which the mammals of the different orders show great diversity in the mode of development, final form of the fetus and placenta, gestation time, etc. (Perry, 1981). Furthermore, the plasticity of the mammalian embryos during the initial period leads them to develop under varying environmental conditions, presumably throughout an adaptive metabolic reprogramming. However, this adaptation can induce immediate effects on the viability and development of the embryo, but it can also have pronounced and persistent effects into the adulthood, including effects on the reproductive performance of successive generations (Ashworth *et al.,* 2009).

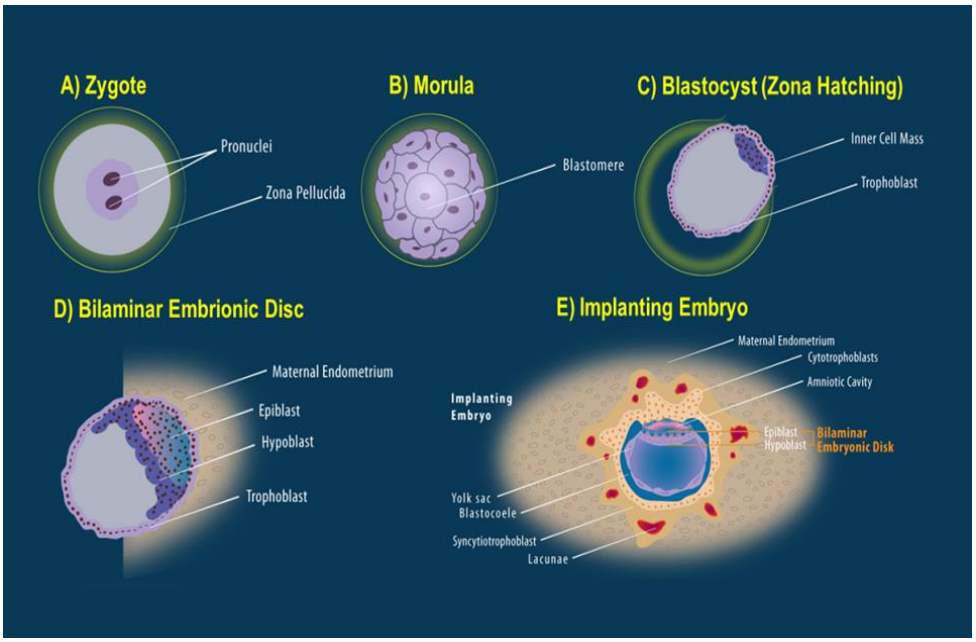

Figure 1. Sequence of events in early pregnancy (Adapted from LifeMap Sciences)

1.2. Implantation phase

The implantation phase begins when the blastocyst reaches the uterus and attaches to the uterine wall (Table 1). The development of the mammalian fetus is dependent upon a successful implantation of the blastocyst (Fleming *et al.,* 2011). This essential step is regulated by the action of the trophoblast cells (TBs), which coordinate the complex processes of embryonic implantation, uterine spiral artery remodelling and initial development of the placenta (Watkins *et al.,* 2015).

Table 1. Timing of implantation events in mammals (Modified from Chavatte & Guillomot, 2007)

Species	Reaches uterus (day)	Implantation (day)	Gestational length (days)
Mice	3	4.5	20
Rabbit	3	6.5	31
Human	3-4	6-7	280
Pig	2	14	115
Sheep	4	15	145
Cow	4	19-20	280
Horse	5-6	30	330

In general terms, at the time of implantation, the blastocyst shows two cell-type populations: cells allocated in the inner cell mass, named embryoblast, and cells differentiated in the outer cell mass, called trophoblast (TB). The TB will eventually give rise to the placenta, while the embryoblast will become the embryo (Figure 1; Sadler, 2011). The mode in which the TBs interact with the uterus for implantation varies among species. Two types of implantation are discerned according to this interaction, superficial and interstitial implantation. In superficial implantation, the TBs attach to the luminal epithelium, while in the interstitial implantation, the blastocyst lodges in groves or folds of the mucosa, invading the endometrium. Superficial implantation occurs in the rabbit, dog, cat and pig and interstitial implantation can be observed in human, mouse and guinea pig (Perry, 1981).

TBs over the embryoblast pole proliferate and produce the chorion, necessary to bring the fetal and maternal blood systems into close contact (Rossant & Cross, 2001). During this process, TBs also differentiate in other cell subtypes or layers. The inner layer, which does not initially contact the maternal tissues, forms the cytotrophoblast (CT; Figure 1) and the outer layer facing the maternal tissue becomes the syncytiotrophoblasts (ST; Figure 1) by fusion of neighboring CT cells (Baergen, 2005). CT cells synthesize anti-inflammatory cytokines and embryonic factors to prevent adverse uterine reaction (Roth *et al.,* 1996). In contrast, ST cells are a continuous specialized layer of epithelial cells, which can be in direct contact with maternal blood in species with interstitial implantation (Perry 1981; Wang, 2010). In these species, ST cells secrete hydrolytic enzymes, which are important for erosion, penetration and digestion of the surrounding tissue, providing nutrients to the embryo and allowing it to embed further into the endometrium (Ferrer-Vaquer & Hadjantonakis, 2013).

The gastrulation begins with the differentiation of the embryoblast into two layers. The layer closest to the TB is the epiblast (Figure 1), which will give rise to the three germ cell layers (ectoderm, mesoderm and endoderm) that will form the embryo and contributes to the formation of different extraembryonic tissues such as the amnion or the umbilical cord. In contrast, the layer bordering the blastocele cavity is the hypoblast (Figure 1), which helps to the development of the extraembryonic mesoderm and YS and will be determinant in the establishment of the axial patterning of the body and nervous system (Ferrer-Vaquer & Hadjantonakis, 2013). Gastrulation

concludes with the appearance of the first somites. Somites are blocks of cells that originate many body structures, such as vertebrae, intervertebral disks or ribs (Resende *et al.,* 2014).

1.3. Placentation

Placentation is the development of the extra-embryonic membranes. This transient organ is involved in essential functions for pregnancy success, as it provides immunological protection against maternal immune system and pathogens (Gude *et al.,* 2004). Moreover, the placenta is an endocrine organ, producing and secreting different hormones (*e.g.* estrogens, progesterone, leptin or placental lactogen) and growth factors (*e.g.* epidermal growth-factor or insulin-like growth-factors I and II), which can act by autocrine, paracrine and endocrine routes. Moreover, this vital and dynamic organ is highly specialized in exchanging gases and nutrients (*e.g.* carbohydrates, amino acids, lipids, vitamins or minerals; Gude *et al.,* 2004).

In general terms, regardless of the structure and interaction with the maternal tissues, in domestic animals, the placenta can be divided in three portions. The portion of the placenta that gives rise to the fetal membranes (chorionic villi, allantois, amnion and vestigial YS) and is in contact exclusively with the fetus is known as the fetal placenta. The portion of placenta that comes in contact with the maternal tissues is the decidua. Part of the decidua is in direct contact with the TBs, while another area creates a shell between the endometrium and the TBs, which grows over the embryo (Khan *et al.,* 2012; Hayashi *et al.,* 2014). The area between the chorionic villi and the decidua is known as the junctional zone, which is the site of growth factor and pregnancy-related hormone production (Georgiades *et al.,* 2002).

Decidualization is the process of differentiation of the spindle-shape stromal fibroblasts of the decidua into the plump secretory decidual cells, which create a pericellular extracellular matrix rich in fibronectin and laminin. The vascular structures formed in the decidua are critical for blood supply to the fetus. The main function of this vascular system is to control maternal blood blow and therefore regulate nutritional and gas exchange. In fact, these vascular structures possess nitric oxide synthases (NOS) receptors, which may facilitate blood supply to the fetus (Khan *et al.,* 2012; Hayashi *et al.,* 2014). Furthermore, the decidua has to allow a very controlled invasion of the TBs. Soon after the placenta is established, it receives blood from both the maternal and the

fetal systems. The utero-placental blood circulation (from maternal to placental compartments) carries nutrient and oxygen-rich blood through the decidual spiral arteries and ultimately directly or indirectly, depending on the type of placentation, to the terminal villi, which contain fetal capillary vessels and STs. The developing fetus is connected to the placenta by the umbilical cord. The feto-placental blood circulation is composed by two circulations, in which the umbilical arteries carry deoxygenated and nutrient-depleted fetal blood and the umbilical vein carries fresh oxygenated and nutrient-rich blood circulating back to the fetal circulation (Wang, 2010).

The relationship between the chorionic fetal membranes and the uterine wall at the histologic site of exchange is the basis for an important classification of the mammalian placenta **(**Figure 2**;** Furukawa *et al.,* 2014):

- Epitheliochorial: This type is considered the most superficial and lacks of significant invasion. No destruction or invasion of the maternal tissues occurs and no layers of endometrium are removed. It is observed in horses, pigs, ruminants, camels, dolphins, giraffes, and whales.

- Endotheliochorial: The maternal uterine epithelium and connective tissue disappear and TBs come into direct contact with the maternal endometrial tissue. This type of placentation is found in dogs, cats, elephants, otters, ant bears and sea lions.

- Hemochorial: Considered as the most invasive placentation, all maternal tissue layers disappear by erosion. Maternal blood comes into direct connection with the chorion. There are different subtypes depending on the number of TBs layers:
    - Hemomonochorial (primates)
    - Hemodichorial (rabbits)
    - Hemotrichorial (rats and mice)

Another important classification of the placenta is based on the developmental structure of the chorionic villi: folded (pigs), lamellar (carnivores), trabecular (some primates), labyrinthine (rodents and lagomorphs), and villous (humans). The villi are where the fetal tissues come in direct or indirect contact with maternal blood and therefore the location for nutrient and gas exchange between mother and fetus (Burton *et al.,* 2006).

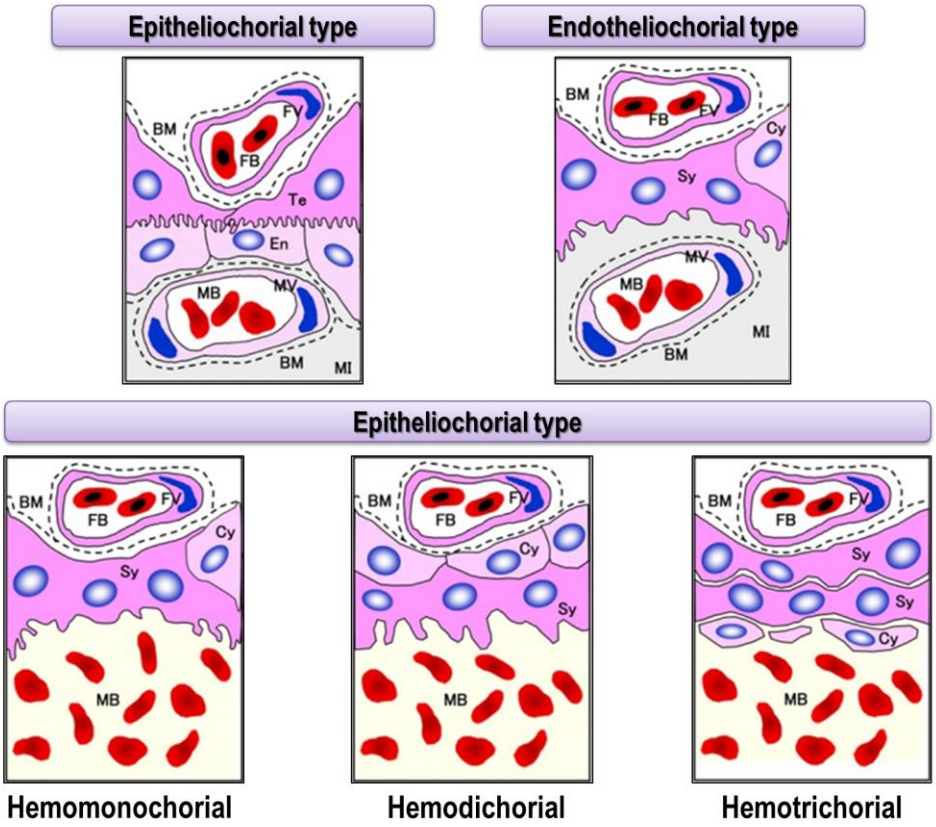

Figure 2. Types of placentation according to the level of infiltration in maternal tissue.

Figure legend: basement membrane (BM); cytotrophoblast (Cy); endometrium (En); fetal blood (FB); fetal vessel (FV); maternal blood (MB); maternal interstitium (MI); maternal vessel (MV); specific trophoblast (ST); syncytiotrophoblasts (Sy); trophectoderm (Te) (Adapted from Furukawa *et al.,* 2014).

1.4. Fetal development

The growth of the fetus can be modulated by different factors (*e.g.* metabolic and endocrine) that interact between maternal, placental and fetal compartments in a complex manner (Sparks & Cetin, 2006). Therefore, a physiological equilibrium of the mother (homeostasis) and a coordinated metabolic response in various tissues or organs to support the new physiological state (homeorhesis or teleorhesis) are required to achieve this cycle of life (Bauman & Currie, 1980; Carlin & Alfirevic, 2008).

This equilibrium is important in all gestational phases. Early pregnancy is the period in which the embryo remains almost microscopic and its energetic demands are low. However, in this initial period, the formation and development of the cell mass, which later will become the infant (embryogenesis), placenta and fetal membranes take place. Mid-pregnancy is the stage of

gestation in which the fetus continues to growth and the development of fetal organ systems (organogenesis) takes place. According to Sparks & Cetin (2006), two types of fetal growth can be observed: 1) qualitatively, in the development and maturation of internal organs, and 2) quantitatively, in increasing fetal size and weight. This last growth pattern is mainly observed during late pregnancy, when fetal body weight gain occurs and the fetus increases its energetic reserves (Gilbert & Jost, 1977).

2. Maternal nutrition & metabolism

Maternal diet must provide sufficient energy and nutrients to cover the high metabolic demands of the conceptus and the basal metabolism of the mother. Moreover, the mother needs to retain certain level of resources to cover the subsequent lactation and future pregnancies (Williamson, 2006; Fowden & Moore, 2012). To cope with the high energetic necessities of the mother and the develop of her offspring, the additional energy requirements of pregnancy result from maternal fat accretion, tissue formation, expansion and oxygen consumption and the needs of the product of conception (fetus and placenta) (Goldberg, 2002; Williamson, 2006). However, determining maternal nutrient needs during pregnancy is complex because nutrient levels in tissues and fluids can be altered by hormone-induced changes in metabolism, shifts in plasma volume and changes in renal function (Picciano, 2003). Notwithstanding, what it is currently accepted is that pregnant diet is very similar to that for the non-pregnant (NP) state (Williamson, 2006).

Maternal metabolism changes during pregnancy to ensure a continuous supply of nutrients to the fetus despite possible intermittent maternal food intake (Butte, 2000). Most of these changes are associated with alterations in maternal lipid profile (Herrera, 2002) and will depend on the level and type of nutrients that the mother and her developing offspring need for each gestational phase. Therefore, from a metabolic point of view, the gestation can be divided in two periods, early pregnancy (anabolic state) and mid-late pregnancy (catabolic state).

Early pregnancy is primarily a time of preparation for the rapid demands for growth that will occur in late pregnancy. Therefore, this period, in which basal glucose and insulin concentrations do not significantly differ from NP values (Catalano *et al.,* 1992), can be considered as an anabolic state in the mother (King, 2000). Moreover, there are remarked changes in maternal fat deposition

(Villar *et al.,* 1992; Hadden & McLaughlin, 2009) in response to maternal hyperphagia (Douglas *et al.,* 2007) and changes in the hormonal milieu (Ryan & Enns, 1988). Specifically, promotion of lipogenesis and suppression of lipolysis in this phase are mediated by changes in insulin, progesterone and cortisol (Hadden & McLaughlin, 2009), which increase hepatic production of triglycerides and enhance removal of these metabolites from circulation (Chandi *et al.,* 2015). As a result, maternal leptin levels (hormone that helps to regulate energy balance) start to rise (Diaz *et al.,* 2014). However, controversial results have been found regarding leptin metabolism in gestation, as this hormone is not only regulated by maternal intake and hormonal milieu, the fetus and the placenta are capable to synthesize this hormone during pregnancy (Hauguel-de Mouzon & Lepercq, 2001; Lepercq *et al.,* 2001).

In contrast, mid-late pregnancy is characterized by lipid mobilization in response to a decrease in insulin sensitivity, raising insulin resistance by 45-70% (Freemark, 2006; Lain & Catalano, 2007; Musial *et al.,* 2016). Thereby, this gestational period can be considered as catabolic state. The resistance to insulin increases hepatic gluconeogenesis (formation of glucose and glycogen from non-glucose precursors via pyruvate) and lipolysis of adipose tissue. Thus, glucose uptake by maternal tissues is decreased, in line with the reduction of maternal energetic storage at the skeletal muscle (Diaz *et al.,* 2014). Thereby, these metabolic changes increase serum maternal glucose and free fatty acid concentrations, allowing for greater substrate availability to the conceptus (Sivan & Boden, 2003; Lain & Catalano, 2007).

2.1. Metabolism of carbohydrates

Glucose is the principal substrate for the placenta and for the fetus (Hay, 2006; Herrera & Ortega-Senovilla, 2010), as fetal gluconeogenesis is minimal (Kalhan & Parimi, 2000). The placenta extracts around 40 to 60% of this metabolite by the uterine circulation, even though it only accounts for about 10% of the total uterine mass (Battaglia & Meschia, 1986; Bauer *et al.,* 1998). Glucose reaches to the fetus after crossing the ST favored by a concentration gradient from mother to fetus. In fact, fetal glucose levels are 15-20% lower respect maternal concentration (Hadden & McLaughlin, 2009; Lager & Powell, 2012). Transport of glucose across the placenta is generally via protein-mediated facilitated diffusion and a number of glucose transporters (GLUTs) are involved (Gude *et al.,* 2004). Moreover, glucose supply is determined by both blood glucose

concentration and uteroplacental blood flow (Baumann *et al.,* 2002). Changes in blood glucose (*e.g.* imbalanced diets or gestational diabetes) or reduction in uteroplacental blood flow (*e.g.* impaired remodeling of the spiral arteries) can alter glucose transfer to the fetus leading to the observation of different fetal growth patterns (Baumann *et al.,* 2002). Thereby, glycemic evaluation in gestation becomes essential. A valuable serum marker of glucose concentration is fructosamine, which reflects the degree of glycosylation of serum proteins, particularly albumin. This metabolite reflects the average blood glucose level over the previous 1-3 weeks (Bor *et al.,* 1999; Nansseu *et al.,* 2015) and is used as an inexpensive method of screening for diabetic pregnancies (Roberts *et al.,* 1983).

2.2. Metabolism of lipids

Cholesterol is a key constituent of cell membranes and the precursor of hormones and metabolic regulators which are necessary for placental steroid synthesis (Butte, 2000; Woollett, 2001; Palinski, 2009). Thus, cholesterol is an important metabolic fuel for the developing fetus. Cholesterol reaches fetal circulatory system after crossing the STs (Palinski, 2009; Bartels *et al.,* 2012). Although fetus and placenta can both synthesize cholesterol (Herrera, 2002), most of the cholesterol is derived from maternal blood via an interaction of circulating cholesterol-carrying lipoproteins [low-density lipoproteins (LDL-c)]. The ST shows LDL receptors which allow the internalization of LDL-c by receptor-mediated endocytosis (Gude *et al.,* 2004). In contrast, high-density lipoproteins (HDL-c) are responsible for returning excess of cholesterol from peripheral tissues back to the maternal liver either in the pregnant or NP state (Kondo *et al.,* 2003). Cholesterol is a fundamental substance during gastrulation as it moderates the sonic hedgehog proteins, which are involved in the early migration and survival of neural crest cells (Ingham & McMahon, 2001). Furthermore, it will contribute to the development of the brain and other fetal tissues (Baardman *et al.,* 2013).

Other important lipids for pregnancy success are triglycerides; in fact, these metabolites represent an important energetic deposit in maternal tissues (Herrera & Lasuncion, 1998). Triglycerides do not cross the placenta in a significant amount (Herrera & Lasuncion, 1998). In fact, maternal triglycerides are hydrolyzed and taken up by the placenta, where soon after they are re-esterificated to provide a reservoir of fatty acids for the fetus. Fatty acids are transported to

fetal liver, where they are re-esterificated, stored and released back into fetal circulation in the form of triglycerides (Herrera, 2002).

3. Malnutrition & conceptus adaptations

The term “maternal malnutrition” focus attention on mothers exposed to inadequate diets due to excesses (overnutrition) or deficits (undernutrition) in their food intake and/or imbalanced diet composition during the period of bearing and/or nurturing their offspring (Blossner & de Onis M, 2005; Black *et al.,* 2008). As a consequence, malnutrition can disrupt fetal growth trajectory which lead to the observation of disorders in fetal growth patterns, such as Intrauterine Growth Restriction (IUGR) giving way to Small for Gestational Age infants (SGA) or growth excess, which implies fetal macrosomia/overgrowth and is defined as Large for Gestational Age (LGA).

Overnutrition and obesity are problems in developed and developing societies, leading to a burn in metabolic diseases such as Type II-diabetes (Gonzalez-Bulnes & Ovilo, 2012; Gonzalez-Bulnes *et al.,* 2015). However, maternal undernutrition is a severe public health issue in emerging countries but also in developed societies. In the developed world, undernutrition is usually associated to esthetical reasons (eating disorders and voluntary caloric restriction; Brett *et al.,* 2014). In emerging societies, undernutrition is related to limited access to high quality foods, wrong traditional habits, food taboos, low social statuses and limited knowledge about the effects of the condition (WHO, 2004). The FAO 2015 Hunger report (FAO, 2015) estimates that about 795 million people are undernourished, mainly in sub-Saharan Africa and South-Central and South-Eastern Asia. Due to the reduced food intake, adult’s height is usually lower than 145 cm and body-mass index of less than 18.5 kg/m². These factors can predispose to adverse pregnancy outcomes, such as cesarean delivery, preterm birth, low birth weight (LBW), and higher rates of morbidity and mortality (Black *et al.,* 2008; Shah & Shah, 2009; Han *et al.,* 2012).

3.1. Placental adaptations

There are increasing evidences in the literature suggesting that the placenta can act as a sensor of the maternal-fetal environment, adapting its morphology (Coan *et al.,* 2010; Zhang *et al.,* 2015), function (Sibley *et al.,* 2005) and cellular composition (Sandovici *et al.,* 2012). These

adaptations contribute to optimize nutrient and gas exchange between the mother and the fetus, in line with the capacity of the mother to allocate her resources and the continuous signaling of the fetus for growth. However, adverse conditions in the intrauterine environment may determine placental morphology during gestation, which will depend on the type and duration of the exposure to the insult (Myatt, 2006). Morphological studies show that physiological remodeling of the maternal uterine vasculature into spiral arteries is deficient in IUGR pregnancies, due to inadequate TB invasion and proliferation (Pijnenborg *et al.,* 2006; Toal *et al.,* 2007). Failure of placental adaptations may result in placental insufficiency and lastly in fetal growth disruption (Sibley *et al.,* 2010). IUGR is often a consequence of this ineffective function of the placenta and can be associated to a higher incidence of perinatal morbidity and mortality, as well as increased risk of suffering from cardiovascular and metabolic diseases in later life.

Placental efficiency, usually evaluated in terms of fetal weight to placental weight ratio, has been used as a proxy of the intrauterine conditions and may indicate the different adaptations that the placenta can perform to favour or constrain allocation of resource according to maternal and fetal signals. In this regard, studies involving embryo transfer between breeds of different size has evidenced that placental size can be modulated according to the genetic potential for growth of the developing fetus and maternal size (Fowden *et al.,* 2009). Although, placental weight usually positively correlates with birth weight, it has been demonstrated that lighter placentas could be more efficient respect those that are heavier, increasing nutrient delivery to support fetal growth under adverse conditions (Fowden *et al.,* 2009). However, this measure not always tracks with placental nutrient transfer capacity estimated per placental surface area (Sferruzzi-Perri *et al.,* 2016).

Placental adaptations in undernourished pregnancies have been reported in different species. In rodents, maternal food restriction by 15-50% reduces conceptus weight by 22-26% respect *ad libitum* pregnancies (Sferruzzi-Perri & Camm, 2016). However, although the exposure to caloric restrictions during the mouse gestation reduces conceptus weight, it has been noticed an upregulation of system A transport and different genes involved in nutrient exchange (*Glut1, Slc38a1, Slc38a2, FABP4, FABP5*) (Ganguly *et al.,* 2012; Chen *et al.,* 2013). These results may indicate that the placenta tries to compensate nutritional deficits by adapting its functional phenotype. Furthermore, in rats, 50% food restriction reduces by 25% conceptus weight and

increase apoptosis in the labyrinth and junctional zones. However, *SNAT4* and *GLUT1* transporters are significantly increased (Belkacemi *et al.,* 2011a, b). Other studies have shown different placental adaptations when oxygen concentration is reduced (hypoxia). In this regard, pregnant guinea pigs exposed to hypoxia showed unchanged or reduced fetal weight, but unaltered placental weight. These changes were associated with alterations in placental function, as evidenced by an increased diffusion capacity, but similar surface area and lower barrier thickness (Gilbert *et al.,* 1979; Bacon *et al.,* 1984). Thereby, maternal environment can shape conceptus phenotype and the placenta tries to adapt its functional and morphological phenotype to help meet the fetal drive for growth (Sferruzzi-Perri & Camm, 2016).

Placental apoptosis has been found to be another factor regulating placental efficiency and fetal growth (Sharp *et al.,* 2010). Apoptosis was first described by Kerr and colleagues in 1972 (Kerr *et al.,* 1972) and refers to a type of cell death. This biological process by which the cell "programs its own death" has been extensively described by Hardy (1999) (summarized in Figure 3). Apoptosis involves a series of consecutive morphologically distinct phases regulated by the effector caspase pathway (Huppertz *et al.,* 2006). This process occurs normally during development, aging and as a homeostatic mechanism to maintain cell populations in tissues (Jaattela, 2002; Elmore, 2007; Soni *et al.,* 2010). In placental tissue, apoptosis is essential for invasion, CT fusion and ST function (Sharp *et al.,* 2010). However, excessive placental apoptotic rates can be observed in cases of IUGR (Smith *et al.,* 1997), preeclampsia (DiFederico *et al.,* 1999; Genbacev *et al.,* 1999; Allaire *et al.,* 2000), hypoxia (Levy *et al.,* 2000) and malnutrition (Belkacemi *et al.,* 2009; 2011a, b). In fact, the expression of p53 and the active form of caspase-3 is upregulated in the placental IUGR villi (Zhang *et al.,* 2015). Thereby, these evidences suggest that these mechanisms of removing unnecessary cells by activation of the caspase pathway are essential for placental development, but exacerbate rates may induce placental dysfunction (Sharp *et al.,* 2010), resulting in a lower placental efficiency to exchange nutrients and gases between mother and fetus.

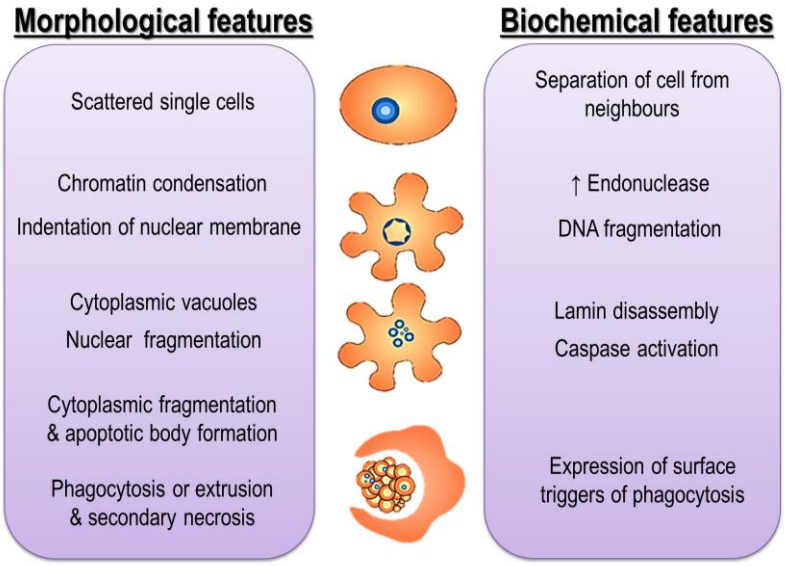

Figure 3. Schematic representation of the morphological and biochemical features of apoptosis in the cell (Modified from Hardy, 1999)

3.2. Fetal adaptations - IUGR

The failure of the fetus to reach its genetically established growth rate is defined as IUGR (Froen *et al.,* 2004; Albu *et al.,* 2014). This impairment in fetal growth is associated with LBW, defined by the World Health Organization (WHO) as a birth weight less than 2,500 grams in the human newborn (WHO, 2004) or weights below the 10th, 5th or 3rd percentile given for a gestational age and sex (Bakketeig, 1998; Lackman *et al.,* 2001). This condition affects around 5 to 10% of the human pregnancies and is the second primary cause of perinatal mortality, after prematurity (Nardozza *et al.,* 2012). However, this pathological condition not only happens in humans, in fact, all eutherian mammals are susceptible to develop IUGR (livestock animals: Wu *et al.,* 2006; dogs: Kliegman, 1989; Zone & Wanke, 2001; rabbits: Thakur *et al.,* 2000; pigs: Campos *et al.,* 2012; Ashworth *et al.,* 2001; non-human primates: Davison *et al.,* 2000; McDonald *et al.,* 2013).

It is important to note that not all small fetuses are IUGR (Alfirevic *et al.,* 2010). In this sense, babies born fundamentally small but that have reached their intrauterine growth potential are known as SGA. This last term refers to a weight for gestation below a specific threshold, which suggests that a percentage of the smallness is due to constitutional or physiological causes and not because of pathological conditions (Figueras & Gardosi, 2011). Thus, IUGR term should be used only to the fetus (Kurjak *et al.,* 2012), such distinction between IUGR and SGA is critical for antenatal as well as postnatal care (Ananth & Vintzileos, 2009).

3.2.1. ***Etiology of IUGR***

The etiology of IUGR is multifactorial and scarcely understood, but it is thought to include a combination of maternal, environmental, fetal and placental factors negatively affecting fetal homeostasis (Table 2) (Sankaran & Kyle, 2009).

Table 2. Causes of IUGR (Modified and completed from Sankaran & Kyle, 2009)

Main causes of IUGR	
Maternal factors	
Undernutrition	Naeye *et al.,* 1973; Bergmann *et al.,* 2008
Maternal low birth weight	Shah & Shah, 2009
Anorexia Nervosa, maternal age, parity	Rusell, 1982; Ong *et al.,* 2002; Solmi *et al.,* 2014
Chronic Hypertension and Pre-eclampsia	Lambert *et al.,* 2014
Systemic Lupus Erythematosus	Feld *et al.,* 2015
Diabetes, renal disease	Grivell *et al.,* 2009; Gutaj *et al.,* 2014
Infections, HIV, Malaria	Sullivan *et al.,* 1999; Venkatesh *et al.,* 2010
Thrombophilic disorders, hypoxemia	Alfirevic *et al.,* 2002, Poudel *et al.,* 2015
Environmental factors	
Drug use- smoking, alcohol, illicit drugs	Ong *et al.,* 2002; Hofhuis *et al.,* 2003; Bergmann *et al.,* 2008; Carter *et al.,* 2013
High altitude, hypoxia, irradiation	Haas *et al.,* 1982; De Santis *et al.,* 2007
Placental factors	
Abnormal placentation, placenta insufficiency	Bekmukhambetov *et al.,* 2016; Kroener *et al.,* 2016
Chronic abruption, infarcts, focal lesions	Ananth *et al.,* 2005; Feist *et al.,* 2015
Chronic inflammatory conditions (villitis)	Feist *et al.,* 2015; Nowak *et al.,* 2016
Single umbilical artery, velamentous cord insertion, placental haemangioma	Hasegawa *et al.,* 2006
Confined placental mosaicism	Simoni & Sirchia, 1994; Wilkins-Haug *et al.,* 1995
Uteroplacental malperfusion	Marcorelles, 2013
Massive perivillous fibrin deposition	Kostadinov *et al.,* 2016
Fetal factors	
Genetic conditions, malformations	Weiner, 1989; Lumley *et al.,* 2001
Intrauterine infections	Grivell *et al.,* 2009
Multiple pregnancy	Houlton *et al.,* 1981

3.2.2. ***Blood flow redistribution in IUGR***

In the situation of chronic fetal hypoxemia or nutrient deprivation, the fetus redistributes its cardiac output to maximize oxygen and nutrient supply to the brain, heart and adrenal glands at the expenses of the growth of other tissues such as lungs, liver, skeletal muscle or skin (Peeters *et al.,* 1979; Jensen *et al.,* 1999; Cohen *et al.,* 2015).

This redistribution is triggered by the carotid body and endocrine and local factors in the fetus, which results in constriction of peripheral circulations and dilation of essential vascular beds (Giussani, 2011, 2016). To sustain the brain's supply of oxygen, blood is shunted from the umbilical vein via the ductus venosus (DV) (Figure 4). The DV dilates and more well-oxygenated umbilical venous blood is shunted to the heart at the expense of hepatic flow (Fu & Olofsson, 2007). This blood flow distribution can be evaluated *in vivo* by the insonation of different blood vessels. The middle cerebral artery (MCA) is the most frequently used due to its intracranial position. In this regard, cerebral hyperperfusion is characterized by a decrease in vascular resistance in the MCA (Fu & Olofsson, 2007) and by changes in the cerebroplacental ratio (CPR), an emerging predictor of adverse pregnancy outcome calculated by dividing the Doppler indices of the MCA by the umbilical cord arteries (UCA) (DeVore, 2015). *Ex vivo* detection of blood flow redistribution can be observed by changes in the brain or liver weight respect fetal weight ratio and brain weight respect liver weight ratio (BLR) (Anderson, 1972; Mitchell, 2001; Marton *et al.,* 2013).

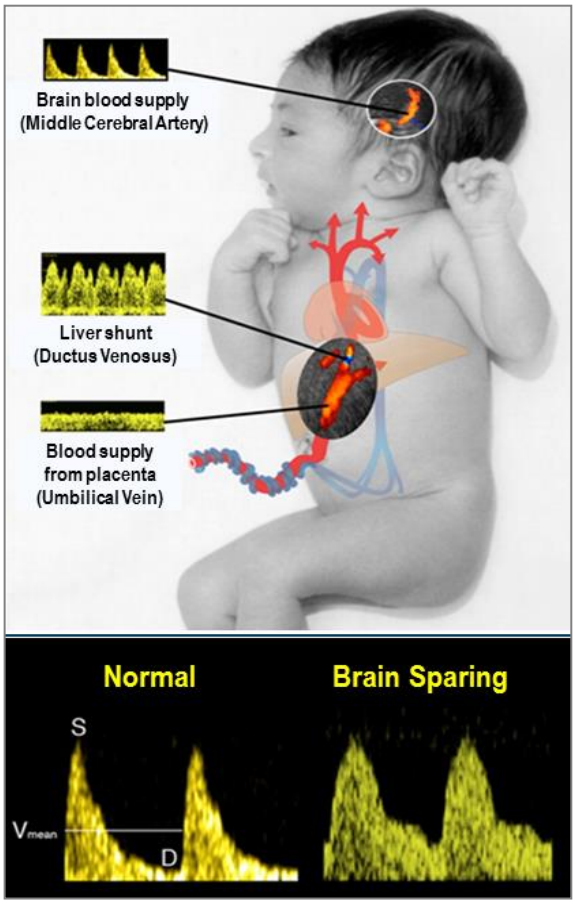

Figure 4. Blood flow redistribution
(Modified from Godfrey *et al.,* 2012)

3.2.3. *Diagnosis of IUGR*

At the ultrasonographic scanning, signs of reduction in fetal biometry such as biparietal diameter (BPD) below the 10th percentile or abdominal circumference below the 5th – 10th percentile are presumptive of IUGR (Bozzetti *et al.,* 2013; Suhag & Berghella, 2013). Moreover, abnormal blood flow in the uterine and fetal blood vessels is often associated to secondary changes in the placental vascular tree (Pomorski *et al.,* 2012) and can be detected by changes in the blood velocity waveform.

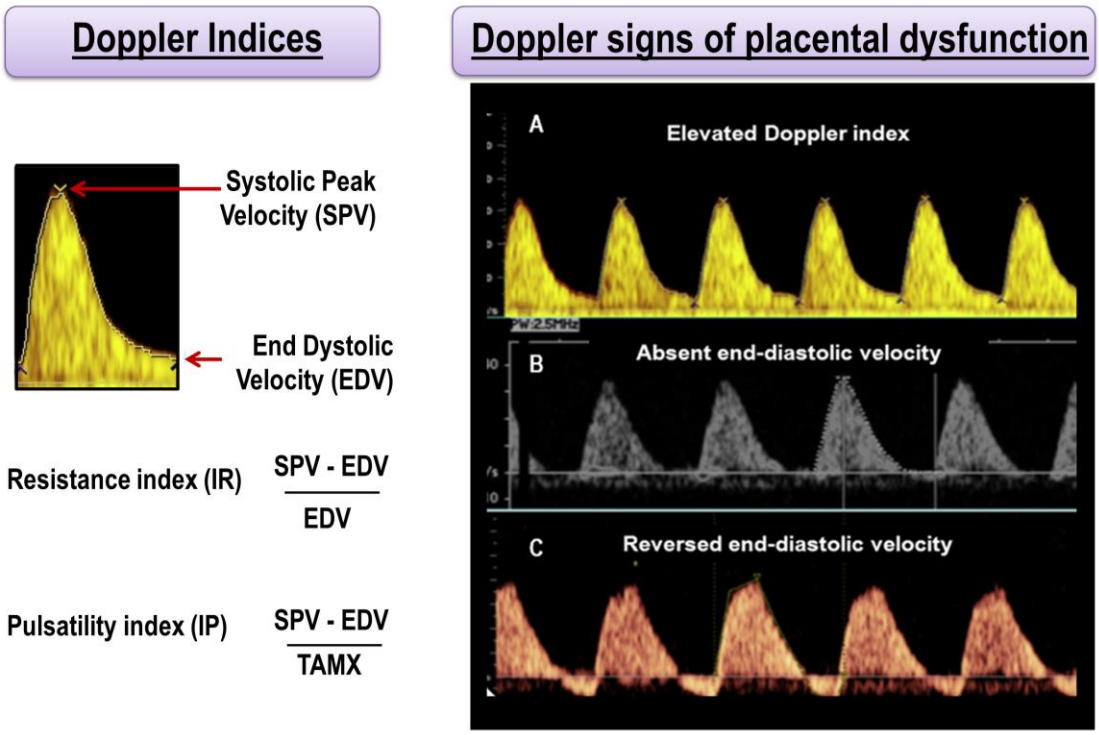

Figure 5. Indices used in obstetric Doppler and arterial flow velocity waveform. Doppler signs of placental dysfunction. A) increased Doppler index associated to placenta insufficiency. B) & C) progression of the sequence of deterioration. Figure legend: systolic peak velocity (SPV), end diastolic velocity (EDV) and time-averaged maximum velocity under the curve (TAMX) (Modified from Baschat, 2011).

Regarding the obstetric examination, the uterine artery (UA) waveform reflects the blood flow resistance in the maternal vascular compartment of the placenta, principally the spiral and placental arcuate arteries (Baschat, 2011). However, despite UA blood flow assessment is commonly used as screening tool to predict adverse outcomes (Kampman *et al.,* 2015), it has been demonstrated to be a poor predictor of IUGR and needs to be combined with additional tests (Cnossen *et al.,* 2008). In this sense, the waveform of the UCA reflects the blood flow

resistance in the placenta and correlates with fetal condition and outcome of pregnancy (Marsal, 2009). In a normal gestation, UCA resistance, determined by mathematical formulas [resistance indexes (IR) and pulsatility (IP); Figure 5] shows a continuous decrease. In contrast, in cases of placental insufficiency, when placental development is affected (≈30% of placental mass damage), end-diastolic velocities in UCA decrease and flow may become absent (Baschat, 2011; Bansal *et al.,* 2016). When this abnormality extends to 60-70%, placenta blood flow becomes reverse (Figure 5; Baschat, 2011). These situations can generate nutrient and oxygen deficits, reduction in fetal growth and lastly fetal death.

3.2.4. ***Types of IUGR***

IUGR can be classified into symmetrical IUGR (type I, proportionate, intrinsic, hypoplastic) and asymmetrical IUGR (type II, disproportionate, extrinsic, hypotrophic). When fetal growth is impaired during the first or second period of pregnancy (early onset growth restriction; Krishna & Bhalerao, 2011), the fetus is more susceptible to develop symmetrical IUGR, characterized by a uniform reduction of the size. This type of IUGR is usually associated to genetic (Trisomies 13, 18, and 21) and infectious factors (cytomegalovirus, toxoplasmosis or rubella virus; Mitchell, 2001; Militello *et al.,* 2009). In contrast, asymmetrical IUGR occurs mainly during the last period of the pregnancy (late onset growth restriction; Militello *et al.,* 2009) and is characterized by a reduction in size of some organs, while other organs remain normal. Insufficient nutritional delivery to the fetus by maternal undernutrition (Bauer *et al.,* 2003) or placental insufficiency (Levene *et al.,* 1985) can predispose to the development of this growth pattern.

3.2.5. ***Therapies for IUGR***

Despite the multiple advances in obstetrics, there is currently non-effective treatment for IUGR available (Sankaran & Kyle, 2009). For many years, non-pharmacological treatments included bed rest, maternal oxygen administration, nutrient therapy or abdominal decompression; however, most of these approaches are not currently on use (Grivell *et al.,* 2009). For all these reasons, clinical trials are currently underway to determine the appropriate therapy that could solve pregnancy complications diseases. In this sense, many of pharmacological candidates have been evaluated, such as Aspirin (Uzan *et al.,* 1991; Leitich *et al.,* 1997; Bujold *et al.,* 2010) or anti-oxidant Tempol (Stanley *et al.,* 2012a).

Nitric Oxide (NO) Therapies: Sildenafil Citrate

NO is produced from arginine by calcium dependent NOS and its biological signaling is mediated by cyclic guanosine monophosphate (c-GMP) (Mergia & Stegbauer, 2016). NO produced by the endothelial and inducible NOS [Endothelial Nitric Oxide Synthases (eNOS) and Inducible Nitric Oxide Synthases (iNOS)] regulates embryo development, implantation and TB invasion (Krause *et al.,* 2011). This potent stimulator of vasodilatation and angiogenesis is required during placental development (Purcell *et al.,* 1999) and is necessary for adapting maternal circulatory system to accommodate the increased blood circulating volume during pregnancy without rising blood pressure (Serrano *et al.,* 2004). In fact, different studies have observed that, during early IUGR conditions, NO concentrations at the fetoplacental compartment are increased as a compensatory mechanism to counteract the deficits in placental blood flow (Lyall *et al.,* 1996; Xu *et al.,* 2000; Pisaneschi *et al.,* 2012). Thus, pharmacological inhibitors of NOS led to maternal hypertension and IUGR (Yallampalli & Garfield, 1993).

Sildenafil citrate (SC, commonly known by its commercial name of Viagra®) has been widely used for counteracting erectile dysfunction problems (Ravipati *et al.,* 2007). This selective phosphodiesterase type-5 (PDE-5) inhibitor prevents the hydrolysis of cGMP and increases NO bioavailability. Thereby, due to its vasodilator and angiogenic properties, this therapy could optimize placental function and alleviate IUGR in at-risk pregnancies (Pyriochou *et al.,* 2007).

The effects of SC have been tested in different animal models of IUGR. SC can improve certain phenotypic parameters of the fetus/newborn (Sánchez-Aparicio *et al.,* 2008; Stanley *et al.,* 2012b; Motta *et al.,* 2015). However, SC efficacy for counteracting LBW is controversial. Studies in sheep with 50% maternal food restriction have shown that this drug increased fetal weight by 14% (Satterfield *et al.,* 2010). However, in other studies this effect had not been found (Ramesar *et al.,* 2010; George *et al.,* 2013; Motta *et al.,* 2015) and may be associated to the animal model and the method for inducing IUGR employed.

In woman, SC has been tested with promising results, improving maternal and fetal blood flow velocimetry and fetal well-being (Lacassie *et al.,* 2004; Lin *et al.,* 2012; Panda *et al.,* 2014; Sun *et al.,* 2014; Trapani *et al.,* 2016). Currently, several clinical trials are underway to further test the usefulness and safety of SC treatments for IUGR (Ganzevoort *et al.,* 2014).

3.3. The Developmental Origins of Health and Disease (DOHaD)

Barker and colleagues firstly described an association between LBW and the higher risk of suffering from non-communicable diseases such as type II diabetes, hypertension or ischemic heart disease in adulthood (Barker & Osmond, 1986; Barker *et al.,* 1989, 1993). These associations, referred to as "Fetal Programming" or DOHaD, suggest that the offspring exposed to a poor nutritional environment or environmental-induced perturbations develop intrauterine adaptations to counteract the deficits in nutrient and oxygen uptake. This re-programmation leads to permanent changes in the development and functionality of fetal organs that can predispose them to suffering from metabolic syndrome or other diseases in later life (Hales & Baker, 2001; Vaag *et al.,* 2012).

The clearest evidence of the relationship between maternal undernutrition, placental adaptations and fetal development occurred during the II World War. The Western regions of The Netherlands were struck by a period of severe food scarcity due to an embargo of food and fuel done by the Germans in response to the Dutch railway strike and their support to the Allied forces (Painter *et al.,* 2005; Franzek *et al.,* 2008). This period of hunger started in the winter of 1944 and finished with the liberation of The Netherlands in May 1945 (Roseboom *et al.,* 2011). Although the famine lasted for 5 months, the restriction affected a previously well-nourished population, so caloric amount significantly decreased throughout the period of the embargo (Painter *et al.,* 2005).

The effects of the Dutch Famine on the developing conceptus changed according to the period of the pregnancy in which the mother was exposed to the famine. Roseboom *et al.* (2011) found that exposure to the famine in any stage of gestation reduced placental area (≈19 cm^2 reduction). The exposure to famine during mid- or late pregnancy also reduced placental length and width, reinforcing the period of TB invasion and spiral artery recruitment as critical points.

Despite placental adaptations, babies exposed to poor nutritional environment during early gestation were heavier than offspring unexposed to famine (Schulz, 2010; Roseboom *et al.,* 2011). However, undernourished offspring evidenced higher rates of obesity, altered lipid profiles, cardiovascular diseases and altered cognitive functions when they grew up (de Rooij *et al.,* 2010;

Schulz, 2010). In contrast, those exposed to the famine only during late gestation were born small and continued to be small throughout their lives, and with lower rates of obesity.

Following the Dutch famine example, the Chinese famine or the Biafran famine in Nigeria generated relevant data about the effects of undernutrition and DOHaD. Most of the studies about the effects of the Chinese famine found associations between undernutrition in utero and increased risk of mental disorders in adulthood (Susser & St Clair, 2013; Huang *et al.,* 2013). In contrast, the Biafran famine studies demonstrated the association of fetal and infant undernutrition and the higher risk of suffering from hypertension and impaired glucose tolerance (Hult *et al.,* 2010).

4. Animal models for studying IUGR

Because of all mentioned above, there is a serious necessity to understand the pathophysiology of IUGR and find an effective therapy to counteract IUGR. Seeing as experimental studies on humans are limited due to ethical constraints, translational research based on the use of animal models have become imperative.

Recently, Swanson & David (2015) reviewed the advantages and disadvantages of the animal model employed for this purpose. Although the closest animals to humans in terms of implantation and conceptus development are the non-human primates (Ramsey & Harris, 1996; Blankenship & Enders, 2003; Fazleabas, 2007; Rutherford, 2012; Su & Fazleabas, 2015), the use of these animals for biomedical purposes presents several ethical constraints and these models are not always available. Thereby, most of the studies have been performed in rodents and sheep (Figure 6). However, in recent years, other animal models such as pigs or rabbits have emerged as suitable models for reproductive studies.

Rodents are candidate models for placental studies due to their similarities in placental development (discoid shape and the exchange interface is hemochorial; Furukawa *et al.,* 2014). In fact, this specie is the most used in research due to its easy management and handling, low costs and the wealthy information available in the literature.

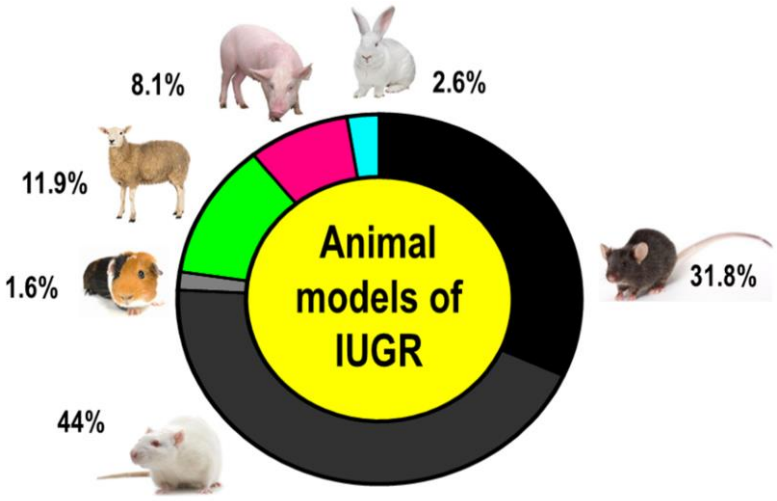

Figure 6. Animal models employed for studying IUGR. Percentages represent the number of articles published over the last ten years in Pubmed database with the keywords "IUGR + animal of research" (From Jorge López Tello).

Regarding the sheep, its main advantage is the occurrence of singleton pregnancies; however, placentation is not closely similar to humans. Other groups have used the sheep for elucidating the effects of high-altitude exposure during pregnancy (a main cause of IUGR by deficiencies in oxygen supply), either by natural conditions (Parraguez *et al.,* 2006, 2011, 2013, 2015; Herrera *et al.,* 2007, 2016) or by using hypoxic chambers (Allison *et al.,* 2016).

In pigs, Gonzalez-Bulnes *et al.* (2016a, 2016b) recently reviewed the use of this animal as a model for pregnancy complications. Due to the intensive selection for prolificacy and the limit of intrauterine space, the pig can be considered as a "natural model for IUGR", as within the litter may coexist animals with an appropriate body weight and other with LBW. However, this variation within the litter is associated with increased preweaning mortality, variable weights at weaning and decreased growth performance (Campos *et al.,* 2012).

4.1. Methods for inducing IUGR

The methods for inducing IUGR can be classified as maternal, placental or fetal approaches, with more specific manipulations at each level (Schröder, 2003). Each method (Table 3) has its own pros and cons when addressing placenta insufficiency and IUGR pathogenesis. In this sense, some authors propose the ligature of uteroplacental vessels as the most precise method, since

this approach restricts both nutrient and oxygen supply. However, this procedure is usually performed on the last few days of pregnancy and is associated with high mortality rates (Eixarch *et al.,* 2011). Genetic knock-out models are useful for determining the role of a genetic allele (*e.g.* eNOS: Kusinski *et al.,* 2012 or Insulin Growth Factor II (Igf2): Coan *et al.,* 2008; Dilworth *et al.,* 2010); however, the costs of producing these animals are usually high and these mutants are not always available in other animal species apart from rodents.

Table 3. Methods for inducing IUGR and placenta insufficiency (Modified from Schroder, 2003)

Maternal intervention
Maternal undernutrition
Protein/caloric, micronutrient deficiency, selected nutrient deficiency
Maternal hypoxaemia
Altitude
Hypobaric hypoxaemia
Normobaric hypoxaemia
- ↓ Maternal fractional inspired oxygen content, intratracheal nitrogen
Carbon monoxide, anaemia, methhaemoglobinaemia, maternal hypertension
Placental intervention
Reduction/restriction of placental growth
↓ Number of implantation sites (hemihysterectomy or carunclectomy)
Maternal hyperthermia
Separation of placentomes during pregnancy
Interference with uteroplacental vasculature - fetoplacental circulation
Partial occlusion of uterine artery
Embolization of uteroplacental circulation
Ligation of interplacental vessels or uterine artery
Embolization via umbilical artery
Fetal intervention
Organ/gland ablation
Hypophysectomie, hypothalamic–pituitary disconnection
Thyroidectomy, radioactive iodine
Pancreatectomy, nephrectomy
Other
Banding ascending aorta, fistula
L-nitro-arginine methyl ester administration
Gene manipulation

As an alternative, diets either by excess or by deficits can induce changes in uteroplacental perfusion, resulting in placental alterations and IUGR (Beck *et al.,* 2000; Sferruzzi-Perri & Camm, 2016). Thus, these diets can be combined with other methods to understand placental adaptations under more stressful situations, such as hypoxia (Higgins *et al.,* 2015) or knock-out

models (Sferruzzi-Perri *et al.,* 2011). Underfeeding programs are based on low-protein diets (Snoeck *et al.,* 1990; Bennis-Taleb *et al.,* 1999; Gonzalez *et al.,* 2016), macro- or micro-minerals deficits (Lisle *et al.,* 2003; Liu *et al.,* 2013), caloric restriction (McNeill *et al.,* 2012; Chen *et al.,* 2013; Ganguly *et al.,* 2014) or global nutrient restriction (Li *et al.,* 2009; Akitake *et al.,* 2015). The effects of these nutritional approaches on maternal physiology, placenta growth and function and offspring outcome (short- and long-term consequences) will depend on different factors:

1) The period of the pregnancy in which the mother and the fetus are exposed
2) The level of restriction applied
3) The capacity of the mother to allocate her resources
4) The capacity of the placenta to adapt and to transfer
5) Fetal requirements for growth
6) Litter size: monotocous *vs* polytocous species

As previously mentioned in this literature review, the time of the pregnancy in which the conceptus is exposed to the restriction in nutrients is critical for its development. In this sense, maternal food restriction (MFR) in rodents is usually associated with placental and fetal weight reduction (Ahokas *et al.,* 1983; Coan *et al.,* 2010; Mayeur *et al.,* 2013; Sferruzzi-Perri & Camm, 2016). However, when mice are exposed to 50% MFR from day 1.5 to 11.5 of pregnancy (early stages of pregnancy; term on day ≈20), placental weight is reduced and other placental phenotype characteristics as well (no data available respect fetal weights) (Schulz *et al.,* 2012). However, these differences are not maintained at the end of gestation and fetal weight is not reduced (Harper *et al.,* 2015). These results, once more, reinforce what previously mentioned about the capacity of the placenta to recover its normal function despite the application of MFR and emphasize its incredible capacity to adapt its function and morphology to support fetal growth demands under stressful situations (Sferruzzi-Perri & Camm, 2016).

In sheep, the effects of MFR on conceptus development are even more inconclusive and may be associated to the reduced litter size (usually considered as monotocus specie) and the length of gestation (pregnancy length ≈147 days). In this specie, the period of maximal placental weight gain occurs in the first half of gestation (from day 30 to 80 of pregnancy) (Heasman *et al.,* 1999) and it has been demonstrated that placentas from mothers restricted between days 28 and 77 of gestation were heavier, but fetal weight was unchanged (Heasman *et al.,* 1998). Moreover, MFR from early to mid-gestation can either enhance or reduce placental growth without affecting fetal

weight (McCrabb *et al.,* 1992). Notwithstanding, when MFR is carried out during late pregnancy, lamb weight is reduced (Gao *et al.,* 2008) and can affect the ontogeny of fetal organ growth and development (Gao *et al.,* 2009).

5. The rabbit as a model

The European rabbit (*Oryctolagus cuniculus*) belongs to the *Lagomorpha* order (Yanni, 2004) and is the second mammalian specie most frequently used for scientific purposes in Europe according to the Seventh Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union (European Commision, 2013).

This animal, phylogenetically closer to primates than rodents (Graur *et al.,* 1996) and considered as a large animal model despite its halfway between small and large animal (Getz & Reardon, 2012), is widely used for biomedical modelling in reproductive sciences because of its countless benefits. Rabbits are easy to handle and they have small life-cycle, with short periods of gestation (30-31 days) and lactation (30 days). The size of the animal allows serial blood sampling (Haneda *et al.,* 2010; Mizoguchi *et al.,* 2010) and imaging of conceptus development can be performed with standard ultrasound equipment (Chavatte-Palmer *et al.,* 2008). Furthermore, hemodynamic changes in the rabbit placenta are comparable with the humans (Fischer *et al.,* 2012; Lecarpentier *et al.,* 2012), with high blood flow velocities in the UCA resembling human values in the second trimester (Polisca *et al.,* 2010).

Overall, rabbits are considered excellent models for toxicology (Carney *et al.,* 2008; Hui *et al.,* 2014; Sweeting & Wells, 2015; Tarazona *et al.,* 2016; Valentino *et al.,* 2016), atherosclerosis (Fan *et al.,* 2015; Tikoo *et al.,* 2015; Baumgartner *et al.,* 2016), orthopaedics (Knapik *et al.,* 2013; Gui *et al.,* 2015; Wei *et al.,* 2015), surgery (Calasans-Maia *et al.,* 2009), diabetes (Duff & Mc MG, 1949; Duff & Payne, 1950; Duff *et al.,* 1954; Haucke *et al.,* 2014; Javadi *et al.,* 2014; Zhou *et al.,* 2015) and reproductive studies (Foote & Carney, 2000; Fischer *et al.,* 2012; Tarrade *et al.,* 2013, 2014; Arias-Alvarez *et al.,* 2016).

5.1. Physiology & characteristics of the rabbit gestation

Rabbits reach sexual maturity at 4-6 months of age (Macari & Machado, 1978; Foote & Carney, 2000). In this animal, ovulation is induced by coitus and due to their high sexual receptivity of the dam, it can encompass the lactation and the subsequent gestation simultaneously (Lorenzo *et al.,* 2014). The gestation lasts 31 days. Rabbit's utero is duplicated and independent, as they present two uterocervicovaginal canals (Weisbroth *et al.,* 1974). This fact facilitates the study of the maternal and fetal genomic interactions by transferring embryos in reciprocal crosses between breeds of different genetic background (Moce *et al.,* 2004).

5.2. Preimplantation phase

Preimplantational events in the rabbit (Figure 7 and Table 4) have been recently reviewed by Fischer *et al.* (2012). Fertilization occurs at ≈10 h *post coitum* (p.c.), the second polar body starts to be visible 14 h p.c. and the embryo genomic activation takes place in the 8- to 16-cell stage. After multiple cell divisions, the morula stage is achieved ≈60 h p.c. From day 4, the rabbit embryo starts to dissolve the zona pellucida and it is replaced by the neozona during day 5 p.c. to promote the implantation process in utero. On this day, the number of cells forming the blastocyst ranges between 1,291 to 9,536. These numbers increase on day 6 p.c. (80,000 to 100,000) according to Fischer *et al.* (2012) review. Another main characteristic of the rabbit embryo is its mucin coat (Seidel *et al.,* 1976; Carney & Foote, 1990), which surrounds the embryo until the hatching of the blastocyst (Denker & Gerdes, 1979). This coat will disappear around day 6 of gestation (Kane, 1975).

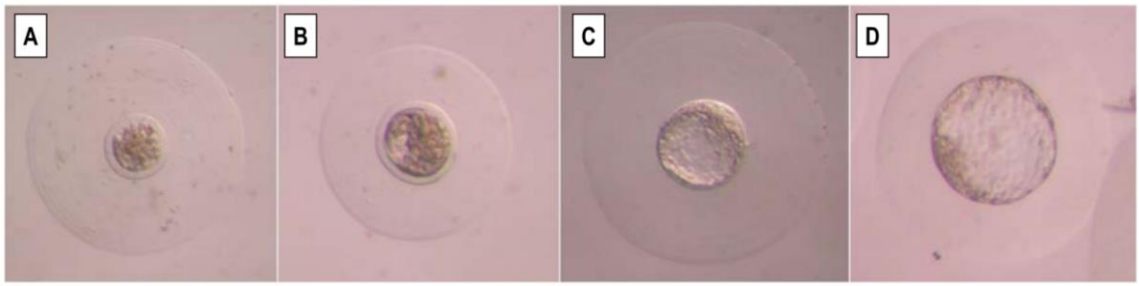

Figure 7. Embryonic development of the rabbit. A) Morula; B) Cavitating late morula; C) Blastocyst; D) Expanded blastocyst (Modified from Arias-Alvarez, 2010).

Table 4. Early pregnancy events in the rabbit (Modified from Weisbroth *et al.,* 1974)

Timetable of: early zygote and blastocyst development	
Hours	Event
0	Coitus
22-26	2-Cell cleavage state
30-34	4-Cell cleavage state
38-42	8-Cell cleavage state
46-52	16-Cell cleavage state
41-75	Morula state
60	Slight changes in peripheral cells destined to become TB
70-80	Blastocyst reaches uterus (depend on length of oviduct)
75	Appearance of blastodermic vesicle, Cleft appears to separate the primitive mass from TB cells
90	Enlargement of blastocyst and increased number of cells in TB
	Disc elongation and growth in posterior direction with slight thickening at anterior end
	Beginning of primitive streak formation with cell condensation on midline
	Primitive streak present in the posterior part of the disc
	Mesoderm begins to grow fanshaped from posterior end of disc

5.3. Gastrulation & implantation phase

As previously mentioned, gastrulation is the process by which the embryo establishes the three germ layers (ectoderm, mesoderm, and endoderm) and, in the rabbit, this process starts on day 6 of pregnancy. Gastrulation is divided in 7 phases (Figure 8), in which implantation takes place on the stage 4, which corresponds to day 7-7.5 of pregnancy (DeSesso, 1997; Nishimura, 2001) and concludes with the appearance of the first somites. These structures appear around day 8.5 of pregnancy in rabbits (Beaudoin *et al.,* 2003) and originate vertebrae, intervertebral disks or ribs (Resende *et al.,* 2014).

mplantation in the rabbit can be divided in two phases and will conclude with the beginning of the placentation at day 8 (Nishimura, 2001). The first phase, also called the “ob-placental implantation”, takes place on day 7 of pregnancy in the antimesometrial side of the uterus. This phase is characterized by the formation of TB knobs, cell fusion to produce ST before attachment to uterine epithelial cells (Hoffman *et al.,* 1999) and form the YS placenta. The second phase occurs in the mesometrial side of the uterus around day 8 of pregnancy and is called “placental implantation”. In this phase, the TBs attach to the placental folds to form the chorioallantoic placenta. In addition, rabbit TBs attach to and invade the endometrium as ST, in a similar pattern to humans and primates. In contrast, the blastocyst attachment of rodents is via CT (Hoffman *et al.,* 1999).

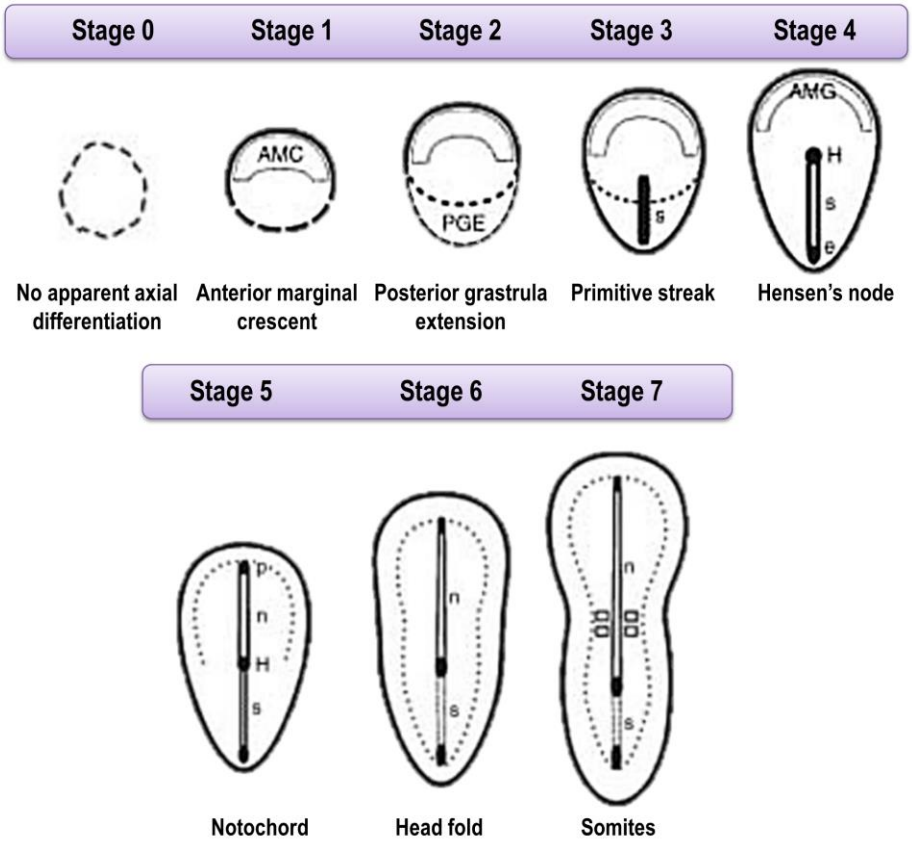

Figure 8. Gastrulation stages in the rabbit embryo (Modified from Fischer *et al.,* 2012)

5.4. Early placentation

The early post-implantation embryonic nutrition in the rabbit embryo, prior to the definitive placentation, is based on maternal histiotrophic nutrition via YS placenta (Furukawa *et al.,* 2014). This type of nutrition is based on maternally derived solutes and macromolecules. It is secreted from endometrial and uterine glands into the space between the maternal and fetal tissues via inverted YS (IYS) (Marshall *et al.,* 2015). After crossing the YS, molecules enter into the exocoelomic cavity (primitive YS) and soon after molecules can be transferred to the embryo via the vitelline vessels. Other possible routes for nourishing the embryo is the periderm diffusion, as the keratinized dermal layer is not yet formed in the embryo (Carney *et al.,* 2004).

The IYS of the rabbit appears at day 9 of pregnancy from the lateral margins of the embryo (Figure 9) and the visceral YS (VYS) becomes increasingly vascularized between days 10 to 12 of pregnancy. The VYS begins to expand wrapping itself around the embryo, concluding this process on day 13 of pregnancy. In contrast, the nonvascular portion of the YS begins to

degenerate by day 10 p.c. (Weisbroth *et al.,* 1974). These events coincide with the onset of chorioallantoic placental circulation (Carney *et al.,* 2004), which is formed from the TBs and allantois and is vascularized by the alllantoic vessels (Mess, 2014).

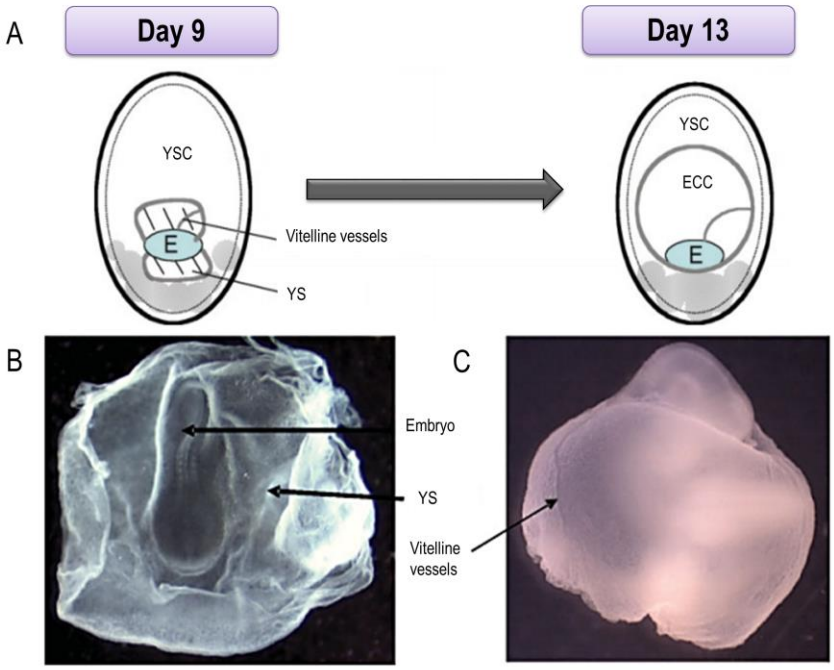

Figure 9. Rabbit's yolk sac. A) Schematic representation of the yolk sac between days 9 to 13 of pregnancy. B) Rabbit embryo and yolk sac on day 9 of pregnancy. C) Rabbit yolk sac on day 12 of pregnancy. Figure legend: embryo (E), exocoelomic cavity (ECC), yolk sac (YS) and yolk sac cavity (YSC) (Modified from Carney *et al.,* 2004).

5.5. Definitive placentation

Rabbits have a hemodichorial and bidiscoid type of placenta (Figure 10) (Furukawa *et al.,* 2014). The establishment and development of the true chorioallantoic placenta occurs during days 10-17 of pregnancy (Weisbroth *et al.,* 1974). Histologically, rabbit placenta presents different compartments, the labyrinth zone (Lz), the junctional zone (Jz) and the decidua basalis (Db), as previously mentioned in this memory, but with particular characteristics.

- Labyrinth zone: According to Furukawa *et al.* (2014), the maternal blood spaces and the fetal blood vessels in the labyrinth zone are separated by two layers of trophectoderm (T) in rabbit (Figure 10). The outer T, which comes into direct contact with the maternal blood, is

comprised of STs, which are joined to the underlying CT layer by adhesion junctions. The inner T is one layer of CT overlying fetal blood vessels. Therefore, rabbit placentation (hemodichorial; bilayer) is closer to humans (hemomonochorial; monolayer) than rodents (hemotrichorial; three layers) (Hayashi *et al.,* 2014).

- Junctional zone: The Jz in rabbit is composed of glycogen cells containing PAS-positive substances. These cells are transiently detected in mid-gestation and disappear before parturition (Furukawa *et al.,* 2014).

- Decidua basalis: The first decidual cells are observed from day 8 of pregnancy in rabbit (Larsen, 1963). The vascular structures of the decidua give off one or more efferent vessels, which rise through the trophoblastic portion of the placenta, branching profusely, especially at the fetal surface (Figure 11) (Carter *et al.,* 1971). As it is demonstrated in humans (Schiessl *et al.,* 2005) and rats (Purcell *et al.,* 1997), rabbit placenta possess NOS receptors, which may facilitate blood supply to the fetus (Khan *et al.,* 2012; Hayashi *et al.,* 2014).

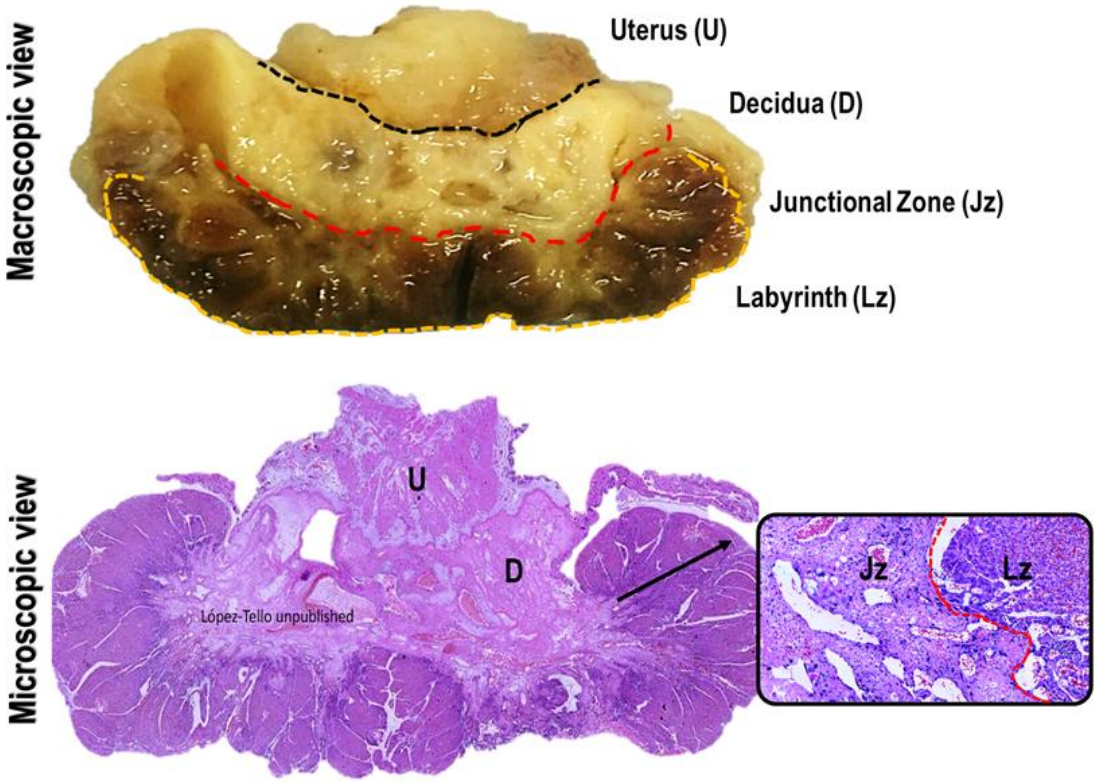

Figure 10. Macroscopic and microscopic images of rabbit placenta at term, day 28 of pregnancy. (Figure belongs to the author, Jorge López Tello).

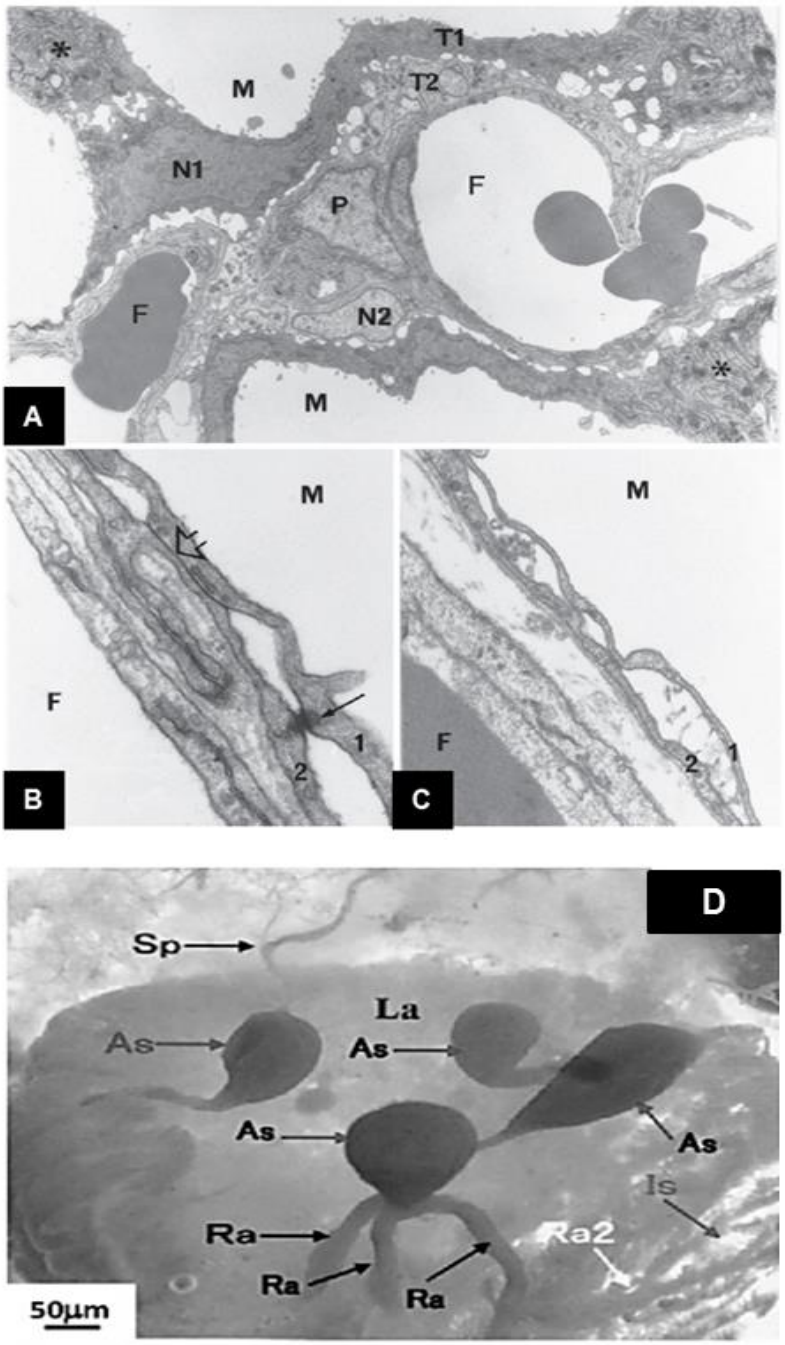

Figure 11. Ultrastructure of the rabbit placenta. (Figures A, B, C) Labyrinth structure of the rabbit placenta. Figure legend: Endoplasmic reticulum (*), fetal blood capillaries (F), fetal pericyte (P), nuclei (N1 and N2), maternal blood space (M), trophectoderm: T1 (syncytium) and T2 (unicellular), gaps (open arrow) and small desmosomal (small arrow) junctions between layers 1 and 2 (Figures A, B & C from Wooding & Burton, 2008). (Figure D) Maternal arterial system of the rabbit placenta on day 18 of pregnancy. Figure legend: Spiral artery (Sp) originated from uterine artery, connects to the arterial sinus (As) and some radial arteries (Ra). Secondary branches of radial artery (Ra2) send small branches into intervillous spaces (Is) (Adapted from Hayashi *et al.,* 2014).

5.6. Fetal development

Rabbit embryo-fetal development has been extensively described by Beaudoin *et al.* (2003). As previously exposed, the first somites appear on day 8.5 and from day 9.5 the cardiac mass as well as the rostral limb bud can be observed (Figure 12). The subsequent limbs will appear on days 10.5 and on day 12.5 they are disposed like wheel rays. Complete fetal appearance is achieved on day 19.5 of pregnancy.

Rabbit's organogenesis, like in other mammalian species, occurs roughly during the second third of gestation. After heart mass, which appears on day 9.5 of pregnancy, the liver and the first intestinal loops appear at 13.5 days. Intestinal growth in the cord concludes on day 17.5 of pregnancy. Abdominal vessels can be observed at 15.5 days and the cecal bud is present outside the abdomen at day 16.5 of pregnancy. On day 18.5, the bowel is returned back in the abdominal cavity and the umbilical ring is closed (Beaudoin *et al.,* 2003). On the other hand, mesonephric ridge, which will give rise to the excretory system of the fetus appears on day 11.5 (Beaudoin *et al.,* 2003). Regarding the nervous system development, the first cerebral vesicle in the rabbit fetus can be observed on day 9.5 of pregnancy. The number of these vesicles increase throughout pregnancy, as on day 11.5 of pregnancy three cerebral vesicles can be identified and on day 12.5 this number increases up to five (Beaudoin *et al.,* 2003). Notwithstanding, the growth and development of the rabbit nervous system (NS) is not limited to fetal life. In fact, in most of the animals, the NS development can be divided in three periods, "prenatal", "perinatal" and "postnatal" (Harel *et al.,* 1972). Rabbits are considered as "prenatal developers" and this characteristic is a principal advantage against rodents ("postnatal developers") for investigating the effects of IUGR on NS, as motor and white matter development is similar to that of humans (Derrick *et al.,* 2004). In rabbits, brain wet weight increases rapidly on the latest 10 days before birth, and continues growing after birth (Figure 12). On these last days of gestation, not only brain weight increases. Rabbit fetal growth is exponential, as it can be observed in the crown rump length (Figure 12). At day 20 of pregnancy, the fetus weighs less than 5 grams and thereafter, it gains about 2 g/day until day 22, followed by a weight gain of 4 g/day until day 24, and about 5 g/day until day 30 (Weisbroth *et al.,* 1974). This increment in fetal weight is in line with the exponential activity of the fetal liver to accumulate glycogen, as it significantly increases after day 25 of gestation (Gilbert & Jost, 1977).

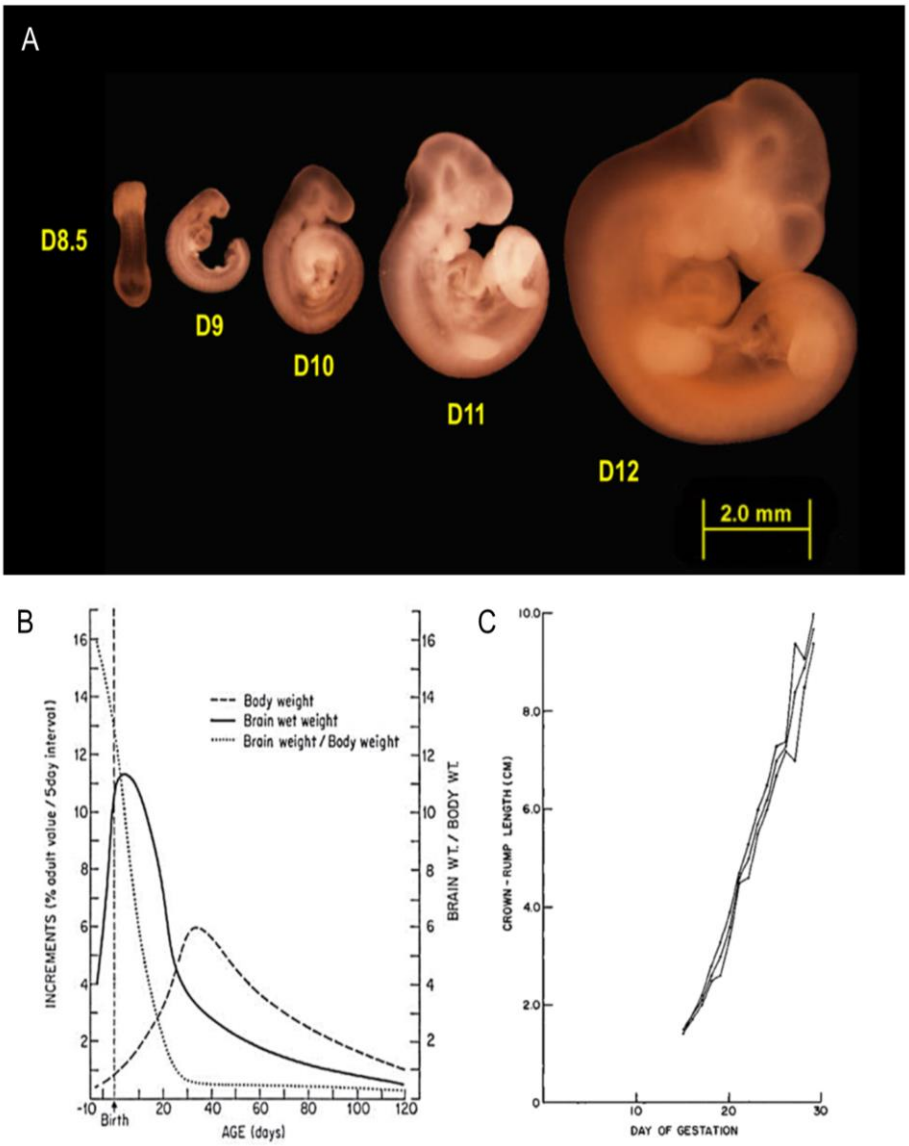

Figure 12. Rabbit fetal growth. (A) Rabbit embryo-fetal early development (Modified from Carney *et al.,* 2007). (B) Rabbit brain and body weight velocity curves (Modified from Harel *et al.,* 1972). (C) Rabbit crown-rump length throughout gestation (Modified from Weisbroth *et al.,* 1974).

5.7. Methods for inducing IUGR and placental insufficiency in rabbits

5.7.1. ***Pharmacological treatment***

In 1996, Losonczy *et al.* (1996) evaluated the role of NO in the pregnancy-induced fall of vascular tone and arterial pressure. However, no data about the effects of this inhibitor on fetus or placental development was available from this study. Years later, the role of L-NAME in rabbit's conceptus growth was determined. Lecarpentier *et al.* (2012) induced IUGR by the administration

of this inhibitor from day 24 of pregnancy. On day 28 of pregnancy, rabbit fetuses treated with L-NAME were reduced in weight, in line with the hemodynamic alterations observed by Doppler. Furthermore, morphological examinations of the placenta revealed a substantial disorganization in the architecture of this organ when L-NAME was administrated. Following this line of research, Tarrade *et al.* (2014) found that the administration of L-NAME reduces volume density of fetal vessels, increases the volume and surface density of the maternal blood space, but does not affect TB volume density or surface density of fetal vessels. Overall, the administration of L-NAME results in placental hypovascularization and IUGR in the offspring. However, the active metabolite of L-NAME (L-NOARG) was found in maternal and fetal plasma and thereby, limitating the use of this approach.

5.7.2. ***Electrical stress & ischemic approach***

Electrical and acoustic exposures during pregnancy are types of stress-induced approaches that can generate placental ischemic tissue and therefore result in hemodynamic alterations and impairment in the adequate feto-maternal exchange (Rosati *et al.,* 1995; Haque *et al.,* 2004).

In 1974, Bal'magiya & Surovtseva (1974) found that the exposure to such stressors during the rabbit embryonic (4-16 days of pregnancy) or partly in the embryonic (16-22 days of pregnancy) periods of development, lead to death of the fetus. In contrast, when fetuses were exposed during the early periods of fetal development (22-23 days of pregnancy), fetal growth and development (organogenisis) were affected. However, when the exposure was carried out during the middle of the fetal period (24-25 days of pregnancy), electrical and acoustic stimulation did not change the weight of the fetuses compared with the control, although the normal weight ratios of the organs were altered. Lastly, stress applied at the end of the fetal period (25-27 days of pregnancy) led to an increase in weight of the conceptus (placenta and fetus) and acceleration of growth of fetal organs.

Rosati *et al.* (1995) found that placental ischemia performed on day 21 of gestation resulted in placental growth restriction and IUGR. Furthermore, fetuses exposed to placental thermal injury had reduced brain and liver weights, with higher BLR, which suggests brain-sparing effect despite no difference in the weights of other organs, such as kidney or heart were found. Another method for inducing placental ischemia and lastly hypoxia is the use of an intraaortic balloon proximal to

the uterine arteries. This procedure, gold approach for studying cerebral palsy, which is a common finding in IUGR (Uvebrant & Hagberg, 1992; Blair & Nelson, 2015), results in cessation of blood supply to the uterus, fetal bradycardia (Tan *et al.,* 1996) and fetal brain injury associated to free radicals (Tan *et al.,* 1996, 1998) and brain edema (Tan *et al.,* 1999). Furthermore, this method of hypoxia–ischemia can generate a broad of long-term consequences in the rabbit offspring, associated to brain white matter injury, such as hypertonia and postural deficits (Derrick *et al.,* 2004; Tan *et al.,* 2005; Derrick *et al.,* 2007; Drobyshevsky *et al.,* 2007).

5.7.3. ***Hypoxia & hyperoxia***

The effects of high altitude exposure (hypoxia; 12.75% O_2) and hyperoxia (70-80% O_2) in pregnant rabbits were determined by Darinskii & Surovtseva (1974). For these purposes, rabbits were placed in a pressure chamber (3 hours/day) from day 23 of pregnancy onwards. On day 30 of pregnancy, the exposure to moderate hypoxia led to marked acceleration of body growth, primarily associated to changes in the growth and development of the skeletal musculature, meanwhile, the mass of the bones remained unchanged. These results contradict Chang *et al.* (1984) study, in which pregnant rabbits exposed to 13.2% oxygen concentration had pups with reduced values of birth weight, placental weight and liver ratio. In contrast, exposure to hyperoxia leds to marked delay in growth of the fetus, which was mainly associated to deficits in skeletal musculature, which suggest IUGR (Darinskii & Surovtseva, 1974).

5.7.4. ***Surgical method***

As previously mentioned in this literature review, the ligature of uteroplacental vessels is a valid approach to induce IUGR. This method could be considered as the most suitable for inducing IUGR, as this technique combines reduction in oxygen and nutrient supply, however, this approach is frequently associated to high mortality rates (Eixarch *et al.,* 2011). As Table 5 depicts, the percentage of vessel ligature vary from 20 to 50%, and is usually performed on the last third of pregnancy (days 21 or 25). Furthermore, the studies performed by using this method are usually designed to unravel the effects that such deprivation in blood supply could generate in fetal brain, heart and kidney development (Table 5).

Table 5. Effects of uteroplacental vessels ligature on conceptus development in rabbits. Table legend: body weight (BoW); brain weight (BW); brain ratio (BR); brain volume (BV); crown-rump length (CRL); ductus venosus (DV); generalized fractional anisotropy (GFA); head circumference (HC); heart weight (HW); middle cerebral artery (MCA); pulsatility index (IP); *postpartum* (PP); †: altered; NDA: no data available.

Author	Day of surgery	% of ligature	Effects	
			Placenta	Offpsring
Harel *et al.*, 1985	26	30	NDA	↑BR + correlations: BoW, BR and HC
Goldin *et al.*, 1987	25	20-30	†Function	†Brain function
Bassan *et al.*, 2000	25	20-30	NDA	↓BoW ↓HC ↑HC/BW ratio ↓Kidney weight ↓Renal glomeruli number
Bassan *et al.*, 2010	25	25	NDA	↓BoW ↓HC ↑HC/BW ratio ↓BW ↓Hemispheral protein level ↓Astrocyte index ↔Total glial cell number
Eixarch *et al.*, 2009	21 25	20-30 40-50	↔Weight	↑Mortality* (↔day25, 20-30%) ↓BoW ↓CRL ↓BW ↑BR
Eixarch *et al.*, 2011	25	40-50	↔Weight	↑Mortality ↓BoW ↓CRL ↓BW ↑BR ↑DV IP ↑Isovolumetric relaxation time ↔MCA hemodynamics
Eixarch *et al.*, 2012	25	40-50	NDA	↓BoW ↓BV ↑BV / BoW ↓Neurobehavioral test †Brain fractional anisotropy
Figueroa *et al.*, 2012	25	20-30 40-50	NDA	↓BoW ↓Renal glomeruli number ↔Renal morphology †Gene expression
Gonzalez-Tendero *et al.*, 2013	25	40–50	↓Weight	↓BoW ↓CRL ↓Abdominal girth ↓HW ↑ HW/BoW †Cardiomyocyte organization
Illa *et al.*, 2013	25	40–50	NDA	↑Mortality ↓BoW †Neurobehavioral test †Brain region infrastructures (↑in grey matter)
van Vliet *et al.*, 2013	25	40–50	NDA	↑Mortality ↓BoW ↓BW †Brain metabolite profile
Batalle *et al.*, 2014	25	40–50	NDA	↓BoW (Until day 70 pp.) ↔BV and fibers at day 70 pp. ↓Brain network infrastructure †Neurobehavioral test ↑Normalized GFA-weighted networks
Torre *et al.*, 2014	25	40–50	NDA	↓BoW ↓HW ↑HW/BoW †Fetal cardiac function ↓Sarcomere length
Simoes *et al.*, 2015	25	40–50	↓Weight	↑Mortality ↓BoW ↓BW ↓BV †Brain metabolite profile
Figueroa *et al.*, 2016	25	40–50	↓Weight	↓BoW Deregulation of kidney vascular activity ↑Oxidative stress damage in kidneys

5.7.5. ***Nutritional managements***

Pregnant rabbits adapt their metabolism in response to their physiological stage (Boyd, 1936; Haugel *et al.,* 1988; Wells *et al.,* 1999; Haneda *et al.,* 2010; Mizoguchi *et al.,* 2010). During the first three weeks of gestation, pregnant rabbits have a positive energy balance and are capable of accumulating fat reserves (+65 g). In fact, pregnant rabbits can increase their food intake by 25-50% (Fortun-Lamothe, 2006). Despite that, during the last pregnancy week, rabbit's energetic balance turns negative, thereby, with possible consequences for fetal growth and the subsequent lactation (Fortun-Lamothe, 2006). Moreover, this adverse balance can reduce maternal reproductive performance and lifespan (Fortun-Lamothe, 2010).

Accordingly, imbalanced diets or malnutrition, either by excesses or by deficits in nutrients intake during the rabbit gestation can generate a broad spectrum of short and long-term effects in the offspring. Furthermore, despite in most of the situations these dietary programs are only performed during the period of gestation. In determined situations, they can alter maternal homeostasis affecting the lactation and therefore having a rebound effect in the growth of the offspring (Hue-Beauvais *et al.,* 2011, 2015). However, as it was previously mentioned in this literature review, the effects of such diets on maternal status, offspring development and growth will depend on the time of application of the diet, the level of fat inclusion or nutrient restriction applied and the response of the dam to such nutritional challenge.

- *High fat diets*

The effects that high fat diets can induce on pregnant rabbit outcome are good examples of the variation that these dietary treatments can have on the development and growth of the offspring. In this regard, Fortun-Lamothe & Lebas (1996) found that the inclusion of fat does not have any effect on mortality rate or in fetal weight. These results are supported by Xiccato *et al.* (1995), who fed pregnant rabbits with a fat inclusion level of 25 g/kg and did not result in variation of litter weight at birth. Moreover, other studies have found that rabbit gestation can be maintained with diets enriched with cholesterol, without affecting maternal prolificacy (Montoudis *et al.,* 1999; Zilversmit *et al.,* 1972; Tarrade *et al.,* 2013; Leveille *et al.,* 2014) or gestational length (Zilversmit *et al.,* 1972).

However, when pregnant rabbits are fed with a high cholesterol supplementation (2%), pregnancy results in miscarriage on day 10 (Montoudis *et al.,* 1999) and may be related to changes in the uterine fluids that could alter blastocyst development, resulting in failure of implantation or poor early placental formation. In this regard, Picone *et al.* (2011) found that cholesterol diets (0.2% of supplement) administered from the prepubertal period altered embryo gene expression in the early stages of the pregnancy (8–16 cell stage). Furthermore, this diet induced IUGR in the offspring and metabolic syndrome in adulthood. Few years later, Tarrade *et al.* (2013) found gene dysregulation at the blastocyst stage in key genes such as *SLC2A1* or *SLC2A3* (solute carrier family genes).

These diets will not only have negative impacts in early embryo development, they are capable for altering maternal, placental and fetal homeostasis (Popjak, 1946; Zilversmit *et al.,*1972; Montoudis *et al.,* 1999, 2003; Marseille-Tremblay *et al.,* 2007; Hue-Beauvais *et al.,* 2011; Picone *et al.,* 2011; Frantz *et al.,* 2012; Cordier *et al.,* 2013; Tarrade *et al.,* 2013; Leveille *et al.,* 2014). In this sense, maternal plasma lipid metabolism is altered and hepatic function can be increased in response to an increment of cholesterol in the diet (Zilversmit *et al.,*1972; Montoudis *et al.* 1999, 2003; Marseille-Tremblay *et al.,* 2007; Tarrade *et al.,* 2013; Leveille *et al.,* 2014). Offspring weight can be reduced, resulting in IUGR (Popjak, 1946; Montoudis *et al.,* 1999); despite in some cases no significant differences in the total lipid content of the offsprings can be observed (Montoudis *et al.,* 1999). Furthermore, offspring metabolism (Montoudis *et al.,* 1999) and liver function can be altered (Montoudis *et al.,* 2003), predisposing the offspring to suffering from late-onset diseases such as atherogenesis (Palinski *et al.,* 2001).

These reductions in fetal weight may are associated to impaired placental phenotype and/or function. Popjak (1946) and Montoudis *et al.* (1999, 2003) found similar altered macroscopic appearance of the placenta and no changes in weight. In both studies, the color of the placenta was altered and may be associated to an excessive cholesterol accumulation within the cells of the fetal capillaries and decidual cells. Tarrade *et al.* (2013) found that this supplementation generated accumulation of lipids in the rabbit placenta at term, with a significant dysregulation of genes involved in transplacental transfers, which lastly affected fetal weight and induced IUGR.

- *Maternal food restrictions (MFR)*

As it has been summarized in Table 6, the application of different food restriction protocols to pregnant rabbits can have important implications for the mother and her offspring outcome. The severity of such effects will depend on the period of gestation in which the restriction is applied, the physiological status of mother and her capacity to compensate the nutritional deficit when the challenge is over. Thus, the age of the mother and the number of parities can have important implications for fetal growth, as body composition, tissue deposition and energy retention can vary in nulliparous rabbit dams (De Blas & Wiseman, 2003).

In the mother, undernutrition can alter body weight (Matsuzawa *et al.,* 1981; Petrere *et al.,* 1993; Cappon *et al.,* 2005; Manal *et al.,* 2010; Nafeaa *et al.,* 2011; Symeon *et al.,* 2015). However, in some occasions, this parameter can be unchanged or even increased as a consequence of the compensatory food intake when nutrients are back provided (Rommers *et al.,* 2004; Menchetti *et al.,* 2015a). In other situations, weight gain can be even higher in dams exposed to the restriction compared to well-nourished females (Petrere *et al.,* 1993). However, it must be noticed that despite body weight is unchanged, some fat storages such as the perirenal fat can be affected (Menchetti *et al.,* 2015a). Regarding other reproductive parameters such as fertility, gestational length, implantation or kindling rates, the application of these feeding regimens does not alter them (Matsuzawa *et al.,* 1981; Rommers *et al.,* 2004; Manal *et al.,* 2010; Menchetti *et al.,* 2015a). However, MFR can result in miscarriage, altered maternal preparturient behavior and metabolic alterations (Matsuzawa *et al.,* 1981; Petrere *et al.,* 1993; Manal *et al.,* 2010; Menchetti *et al.,* 2015b). Finally, milk production can be reduced (Menchetti *et al.,* 2015a) or unchanged (Manal *et al.,* 2010).

Changes in perinatal and postnatal growth as well as in mortality rates can be observed when MFR is applied in rabbits (Table 6). However, these effects depend on the level of restriction and the gestational window in which the restriction is conducted. As an example, a reduction of 1.32-1.35 based on maternal requirements applied in early stages of the pregnancy does not affect litter size, although body weight of the offspring can be either unchanged or increased (Rommers *et al.,* 2004; Manal *et al.,* 2010). Thus, the aforementioned level of restriction reduces mortality rate at birth, may be associated to an excessive fatness of the well-nourished females rather than a direct effect of the MFR. In contrast, a significant reduction of food intake (administration of ≤60

Table 6. Effects of MFR on maternal homeostasis, placental development and perinatal and postnatal offspring growth. Table legend: altered (†); body weight (BW); delivery (D); maintenance requirement (MR); no data available (NDA); non-essential fatty acids (NEFA); perirenal fat weight (PFW); triiodothyronine (T_3); weight (W).

	Challenge		Effects			
	Restriction	**Period**	**Mother**	**Placenta**	**Perinatal offspring**	**Postnatal offspring**
Matsuzawa *et al.,* 1981	Groups: 150 g/day; 60 g/day; 20 g/day	6-20	†BW ↑Miscarriage (20g/day) ↔ Implantational rate	↓W (20g/day)	↑Mortality (60-20g/day) ↓BW (20 g/day)	NDA
Petrere *et al.,* 1993	Groups: 150g/day; 75 g/day; 15 g/day + *ad libitum* refeeding	6-18	†BW ↑Miscarriage (15 g/day)	↔W	↔Postimplantation loss, ↔Mortality rate ↓BW (male15g/day) ↔BW (female15g/day) ↑Malformations (15g/day)	NDA
Rommers *et al.,* 2004	1.35 of MR + *ad libitum* refeeding	0-10	↔BW; Compensatory feed intake ↔Gestational length	NDA	↔Litter size ↓Mortality rate ↔BW	NDA
Cappon *et al.,* 2005	Groups: 150g/day (control); 110 g/day; 75 g/day; 55 g/day; 35 g/day; 15 g/day + control diet refeeding	7-19	↓BW (≤ 55 g/day)	NDA	↓BW (≤ 75 g/day) No external or visceral malformations ↑Unossified bones (≤ 75 g/day)	NDA
Manal *et al.,* 2010	1.32 of MR + *ad libitum* refeeding	0-10 0-15 0-20	↑BW; Compensatory feed intake ↔P4 concentrations ↔Fertility, kindling rates †Preparturient maternal care (nest) ↔Milk production ↔Gestational length	NDA	↑BW at birth ↓Mortality rate	↑BW R15, R20 (weaning)
Nafeaa *et al.,* 2011	60% restriction (111g/day)	0-15 15-D	†BW ↓Serum total proteins	NDA	↔Litter size ↓BW at birth (15-Delivery) ↑Mortality rate (15-Delivery)	↓BW at 21st (15-D) ↑BW at 21st (0-15)
Menchetti *et al.,* 2015a	90g/day *vs* 130 g/day (control diet; 30.8% R) + 130 g/day refeeding	0-9 9-18 19-28	↔BW kindling ↔ Fertility ↓PFW (9-18; 19-28 groups) ↓Milk production ↔BW weaning	NDA	↔Gestational length ↔Litter size ↔BW at birth †Mortality rate: ↔0-9; ↓9-18; ↑19-28	↔Litter size weaning ↔BW at weaning †Mortality rate: ↔0-9; ↑9-18; ↑19-28
Menchetti *et al.,* 2015b	90g/day *vs* 130 g/day (control diet; 30.8% R) + 130 g/day refeeding	0-9 9-18 19-28	†[leptin, insulin, T3, NEFA, glucose]	NDA	NDA	NDA
Symeon *et al.,* 2015	50% of MR	7-19 20-27	↓BW (20-27)	NDA	↓Mortality rate † BW at birth (20-27)	↔BW, feed intake ↔ carcass ↔ meat quality
Goliomytis *et al.,* 2016	75% of MR	7-26	NDA	NDA	↓BW ↑Mortality ↔Gestational length	↓Litter weight (kindling) ↑Mortality ↔ Performance & meat quality

g/day) ranging from day 6 to 20 of pregnancy, a part from resulting in miscarriage, can affect body weight of the newborns and increase rates of malformations (Matsuzawa *et al.,* 1981; Petrere *et al.,* 1993; Cappon *et al.,* 2005). The postnatal growth of these animals can be also affected in different manners according to the period of gestation exposed to the restriction. In this sense, exposure in early pregnancy can increase body weight (Manal *et al.,* 2010; Nafeaa *et al.,* 2011). In contrast, individuals exposed during late gestation can reduce their body weight at weaning (Nafeaa *et al.,* 2011). Regarding the productive aspect of these animals, despite body weight of these animals at birth may be altered, studies evaluating the quality of their product have observed that meat quality is unchanged (Symeon *et al.,* 2015; Goliomytis *et al.,* 2016), suggesting a possible catch up during lactation.

Finally, studies involving placental formation and function in rabbits exposed to MFR are scarce. It has been observed that MFR does not result in a compensatory increased weight of this organ, as it has been observed in other animal species (Heasman *et al.,* 1998; Langley-Evans *et al.,* 1996). In fact, placental weight can be either reduced or unaltered (Matsuzawa *et al.,* 1981; Petrere *et al.,* 1993; Eixarch *et al.,* 2011), reinforcing the inconclusive data exposed in previous sections.

6. The rabbit as a livestock animal: importance of food restriction on fetal growth

Apart from the biomedical use of the rabbit, this animal can be categorized as a farming animal, mainly for meat and fibre production (Allain & Renieri, 2010). In fact, the livestock production of the rabbit reaches to 1.8 million metric tons a year, which is mostly concentrated in Asia (48.8%), Europe (28.4%), America (18.1%) and Africa (4.7%) (Dalle Zotte, 2014). In livestock animals, the implications of DOHaD are outstanding for farm management (Chavatte-Palmer *et al.,* 2015, 2016; Gonzalez-Bulnes *et al.,* 2016b), since animals affected by IUGR are penalizing profitability.

Rabbit livestock production is based on a cycled production in which this animal is normally under 42-day semi-intensive rhythm [Artificial insemination performed on day 11 *post partum* (p.p.) and weaning is done at day 35 p.p. (Lorenzo *et al.,* 2014)]. This rhythm can affect the critic energetic balance of the dam and may jeopardize the success of the subsequent gestations, as these females are always either pregnant, suckling or combining both processes within the same productive period (Figure 13) (Goliomytis *et al.,* 2016). Consequently, the administration of an adequate nutrition will be essential for maintaining the health of the dam, but also for the economic viability of the farm and for the future success of the rabbit industry.

Figure 13. Rabbit dam combining lactation and subsequent gestation.

In commercial rabbit production, most of the productive costs are associated to animal feed, which can be more than 60% of the global costs of the farmer (De Blas & Wiseman, 2003). In addition, the low price of the rabbit at the first industrial sector and the drop of sales had meant that many farms were forced to close down, especially in the Mediterranean area. In the regular management of the rabbit farm is frequent to feed dams *ad libitum* or to appetite after mating. Thus, these types of feeding programs are also maintained during gestation and lactation (Nafeaa *et al.,* 2011). However, these practices are expensive and in some cases, they are not fully recommendable since they can increase the fatness of the animal, especially when they are not pregnant and need to wait for the next insemination in the following cycle. This may result in

higher risk of health problems in the subsequent cycles, such as infertility or parturition problems (Fortun-Lamothe & Lebas, 1996).

Although in previous sections of this introduction it has been listed the different effects that nutritional imbalances can generate on placental and fetal development, from a livestock perspective, the application of a moderate MFR strategy in specific and controlled periods of the gestation could be a practical nutritional approach, provided that fetal development is not prejudice. Thereby, this management could reduce productive costs, avoid maternal fattening and diminish mortalities at parturition. However, it is important to study the adequate threshold of MFR and the gestational windows in which this feeding program is applyied.

Chapter 2: Objectives & thesis outline

Objectives & thesis outline

The main objective of the thesis was to determine whether moderate maternal food restriction protocols applied in specific time windows of the rabbit gestation could induce IUGR in the rabbit offspring. We also sought to determine whether Sildenafil Citrate could be a suitable therapy to counteract placental insufficiency and IUGR induced by maternal food restriction. In virtue of the double function of the rabbit, biomedical and productive, we addressed these objectives with these two perspectives in mind.

These objectives were evalutated in three studies, which are:

1. ***"Characterization of early changes in fetoplacental hemodynamics in a diet-induced rabbit model of intrauterine growth restriction"***

To determine the suitability of the rabbit as a maternal undernutrition IUGR model by fetal ecography and Doppler ultrasonography of conceptus hemodynamics and characterization of offspring growth at birth.

2. ***"The effects of sildenafil citrate on feto-placental development and haemodynamics in a rabbit model of intrauterine growth restriction"***

To evaluate the effects of Sildenafil Citrate administration on fetoplacental hemodynamics, conceptus development and offspring growth at birth in the rabbit model of IUGR induced by maternal food restriction.

3. ***"Competition for materno-fetal resource partitioning in a rabbit model of undernourished pregnancy"***

To determine the effects of different maternal food restriction protocols on maternal metabolism, histopathological study of the placenta and fetal development.

Chapter 3: Results

Characterization of Early Changes in Fetoplacental Hemodynamics in a Diet-Induced Rabbit Model of IUGR

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ORIGINAL ARTICLE

Characterization of early changes in fetoplacental hemodynamics in a diet-induced rabbit model of IUGR

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Intrauterine growth restriction (IUGR) is associated with adverse perinatal outcomes and late-onset diseases in offspring. Eating disorders, voluntary caloric restriction and maternal undernutrition can all induce IUGR but a relevant model is required to measure all its possible consequences. In this work, pregnant rabbits were used as an IUGR model. Control females ($n = 4$) received *ad libitum* diet throughout pregnancy, whereas underfed females ($n = 5$) were restricted to 50% of their daily requirements. Offspring size was measured by ultrasonography and *in vivo* at birth. Hemodynamic features of the umbilical cords and middle cerebral arteries (systolic peak velocity, end diastolic velocity, pulsatility index and resistance index) were characterized by Doppler ultrasonography. At day 21, maternal underfeeding resulted in a significant reduction of fetal size (occipito-nasal length). At birth, the size of kits from the underfed group was significantly lower (lower crown-rump length, biparietal and transversal thoracic diameters) and a reduced weight with respect to the control group. Feed restriction altered blood flow perfusion compared with does fed *ad libitum* (significant higher systolic peak, time-averaged mean velocities and lower end diastolic velocity). Fetuses affected by IUGR presented with compensative brain-sparing effects when compared with the control group. In conclusion, the present study supports using rabbits and the underfeeding approach as a valuable model for IUGR studies. These results may help to characterize IUGR alterations due to nutrient restriction of mothers in future research.

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Key words: brain sparing, Doppler ultrasound, intrauterine growth restriction, pregnancy, rabbits

Introduction

Nutritional imbalances in developed societies are traditionally linked to excessive food intake, but currently the incidence of eating disorders and voluntary caloric restriction for aesthetical reasons are increasing.[1] These circumstances, besides the traditional high prevalence of maternal undernutrition in developing countries,[2] make research on the effects of undernutrition in the reproductive outputs of the exposed individuals necessary. Maternal undernutrition is considered a major cause of intrauterine growth restriction (IUGR).[3] Other maternal causes of IUGR described in the bibliography are hypertensive disorders of pregnancy, diabetic vasculopathy, chronic renal disease, collagen vascular disease and thrombophilia.[4]

IUGR is defined as the failure of the fetus to reach its genetically established growth rate. It is annually related to 800,000 neonatal deaths worldwide[5] and can be classified as 'symmetrical' or 'asymmetrical', with different degrees of severity. Symmetrical IUGR is characterized by a uniform reduction in size of the fetus and its organs and is associated with genetic and infectious factors. Asymmetrical IUGR, however, is characterized by a reduction in size of some organs, while other organs remain normal. It is mainly related with insufficient nutritional delivery to the fetus by maternal undernutrition or placental insufficiency. Predisposing factors that alter the fetal growth trajectory are a detrimental maternal nutritional status and inadequate diet during pregnancy.[6,7] Moreover, maternal undernutrition may affect placental growth and function, thus decreasing nutrient availability to the fetus and as a result affecting neonatal size.[8,9]

Furthermore, a clear relationship between IUGR infants and metabolic, cardiovascular and neurological pathologies in adulthood has been documented.[10,11] The association between inadequate intrauterine nutrition with the occurrence of IUGR and disease risk at postnatal life gave basis to the concept of the Developmental Origins of Health and Disease, formerly known as fetal programming or the Barker hypothesis.[12] This concept predicts that specific situations experienced in early life may increase the risks of suffering late-onset diseases.[13] However, it is unclear how the fetus responds to this prenatal environment to increase its chances of survival.

There is a serious necessity to address the potential long-term effects of undernutrition on the offspring via preventative

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procedures and focused treatments. Seeing as experimental studies on humans are limited due to ethical constraints, translational research based on the use of animal models have become imperative. This is verified by the fact that around three quarters of IUGR experiments have been performed in rats and mice.[14] However, in the recent years, the rabbit has been established as one of the most amenable animal model for reproductive studies.[15] In brief, ovulation in rabbits is induced by coitus, resulting in a precise pregnancy and embryonic age. Implantation starts on day 6–7, completing the chorioallantoid placentation around day 9.[16] After 31 days of pregnancy, females are able to deliver more than eight fetuses, hence enabling a high number of samples per female when compared with other mammal models, reinforcing the 3 R concept on Animal Experimentation.[17] The placental structure of the rabbit is hemodichorial (two cellular layers of chorion between the maternal and the fetal blood) and so it is closer to the human structure than that of other laboratory animals, like rodents (hemotrichorial). Moreover, the similar development of the brain in rabbits and humans (white matter maturation process starts during the intrauterine period[18]) favours the use of rabbits as an useful model to study perinatal processes of brain development.[19] Furthermore, the fetal size in rabbits, significantly larger than in rodents, allows accurate fetal measurements performed with common veterinary ultrasound equipments.[20]

Most methods used to induce IUGR are based on nutrient restriction or surgical modification.[21,22] Some authors propose the ligature of uteroplacental vessels as the most precise method, since restrict both nutrient and oxygen supply; however, that procedure is performed on the last few days of pregnancy and is associated with high mortality.[22] As an alternative, underfeeding regimes are an easier way to induce IUGR in fetuses and are also useful for understanding the pathology of IUGR and its connection to inadequate dietary habits. The availability of a reliable animal model to study fetoplacental hemodynamics in IUGR pregnancies would be highly beneficial in order to develop diagnostic, preventive and therapeutic strategies in humans and animals. Our hypothesis is that the rabbit is a suitable model for the study of the IUGR syndrome caused by maternal undernutrition.

Therefore, the present work aimed to evaluate the suitability of the rabbit as a maternal undernutrition IUGR model. Suitability will be determined by ultrasonography and direct measurements of effects of such perturbations on the development of the offspring and characterizing fetal hemodynamic features at 70% pregnancy.

Methods

Animals and husbandry

The experiment involved a total of nine multiparous pregnant New Zealand × California rabbits (*Oryctolagus cuniculus*) in optimal body conditions and with similar parity. Animals were maintained in individual flat-deck cages under approved animal husbandry conditions with a constant photoperiod of 16 h of light per day, a temperature of 18–22°C and a relative humidity of 60–75%, since these are the normal husbandry conditions for rabbits.[23] The research was performed under a Project License approved by the UPM Committee of Ethics in Animal Research, in agreement with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in research.

Rabbits were inseminated using fresh diluted semen (commercial extender, MA 24; Ovejero, León, Spain). Each dose contained at least 25 million spermatozoa in 0.5 ml of diluent (Magapor S.L., Zaragoza, Spain). Ovulation was induced with gonadoreline at the time of mating (20 μg/doe, i.m.; Inducel-GnRH; Ovejero). Pregnancies were diagnosed by ultrasonography at day 9 after artificial insemination (Sonosite S-Series; SonoSite Inc., Bothell, WA, USA).

Underfeeding model

At day 9 of pregnancy (this is 30% of the pregnancy length in rabbits, with a total pregnancy period of 31 days), when the trophoblast begins to tap maternal blood vessels,[24] nine pregnant rabbits were randomly distributed in two different groups adjusted for similar weight and therefore feed intake consumption. Females were fed the same standard diet (16% crude protein, 37% crude fiber, 3.7% fat and 2400 kcal/kg of digestible energy) fulfilling either their daily maintenance requirements for pregnancy (control group, $n = 4$) or only 50% of that requirements (underfed group, $n = 5$). The diet of each female was adjusted to its feed intake, estimated 2 weeks before the experiment began. Every day, the individual feed ration was weighed and given to the rabbits in their cages. In order to determine changes in maternal body weight, does were weighed on the day of the artificial insemination and after delivery. After insemination, until the end of the trial, maternal feed consumption from the control group was estimated weekly to evaluate a possible increase of the intake during pregnancy.

Conceptuses study

Ultrasound evaluations were performed in all rabbits on the 21st day of pregnancy (≈70% of the total pregnancy length). At this stage, fetal viability is critical since a significant change in the uteroplacental blood flow takes place.[25] This is, when the irrigation of the placenta becomes more abundant than that of the uterus, in order to cover the needs of the developing conceptuses. Moreover, at this moment, organogenesis is assumed to be achieved.[22] Rabbits were shaved in the abdominal area and manually restrained in dorsal recumbence, without anesthesia to avoid any effect on the heart rate or blood flow during the observations. All the females had been handled and restrained before the experimental phase, which diminished animal stress. The same blind operator scanned all rabbits using a Vivid-I ultrasound machine (General Electric, Fairfield, CT, USA)

equipped with a multifrequency (8–12 MHz) lineal array probe. Scans were recorded using the 'cine-loop' option; this allowed animals to be released quickly, minimizing the restraining time. A complete scan exploration did not last >20 min/female.

To visualize the uterine horns and fetuses on the transverse, frontal and sagittal planes, rabbits were scanned by placing the transducer on one flank of the rabbits and moving it to the opposite flank. Four fetuses were selected at random in each female to minimize individual effects. Hence, 16 fetuses were analyzed in the control group and 20 in the underfed group, but the four fetuses measured per mother were averaged to result in one data point. Measurements were obtained with built-in electronic calipers on the cine-loop once the complete examination was recorded. Since the size of the fetus was too large for viewing the entire body-length at this pregnancy stage, measurements included the thoracic diameter, the occipito-nasal length and the biparietal diameter (Fig. 1).

Immediately after birth, all the kits were classified as newborns or stillborns. Only newborn were measured. Using a slide caliper, we also determined biparietal diameter (from one parietal eminence to the other), thoracic diameter (adjusted between underarms) and crown-rump length (maximum distance from crown to tail basis; see Fig. 2).

Doppler evaluation of fetal hemodynamics

At the 21 day of pregnancy, blood flow parameters from the umbilical cord arteries (UCAs; Fig. 3a) and from the middle cerebral artery (MCA; Fig. 3b) were determined in the same fetus in which the body size was ultrasonically evaluated. Briefly, after identifying the vessels with color Doppler (UCAs were found at the free-floating umbilical cord proximal to the placental insertion whereas MCA were located after the Circle of Willis identification), the sample pulsed Doppler gate was placed over the vessels. Then, the waveforms of three consecutive cardiac cycles in each vessel were recorded, disregarding views with insonation angles between 20–50°. Measurements were obtained once the entire examination was recorded and included resistance index (RI), pulsatility index (PI), systolic peak velocity (SPV), end diastolic velocity (EDV) and time-averaged mean velocity (MV), measured at UCA and MCA (Fig. 3). Finally, the cerebroplacental ratios (i.e. the ratios between MCA and UCA values) for RI, PI, SPV, EDV and MV were also calculated.

Data analysis

Statistical analysis was performed with the Statistical Analysis System Software (SAS, 1990). Groups were compared using an analysis of variance (ANOVA) with the treatment (control or underfeeding diet) as the main source of variation and the number of fetuses per doe as a covariate. Mortality rate was assessed with a χ^2 test. If significant main effects were detected, a Student's *t*-test was used to compare averages among groups ($P < 0.05$). For assessing morphometric data, the four fetuses measured per mother were averaged to result in one data point. The relationships between morphometric changes and hemodynamic parameters were measured by Pearson correlation procedures. Regarding maternal body weight, statistical analysis was performed with an ANOVA and the weight at the beginning of the trial as a covariate. Feed intake during pregnancy in the control group was assessed by a repeated measure analysis with time as the main effect. All data were reported as means ± S.E.M. and probabilities were considered significant at $P < 0.05$.

Results

Maternal status

Regarding maternal body weight, no differences were found at the day of the artificial insemination (4.30 ± 0.008 *v.* 4.34 ± 0.010 kg, in control and underfed groups, respectively) nor after delivery (4.44 ± 0.037 *v.* 4.42 ± 0.081 kg). Similarly, feed intake did not change significantly along pregnancy in the control group (216.16 ± 10.60 g/day in the 1st week; 236.33 ± 18.08 g/day in the 2nd week; 218.85 ± 22.14 g/day in the 3rd week; 176.31 ± 22.14 g/day in the 4th week of pregnancy; $P = 0.22$).

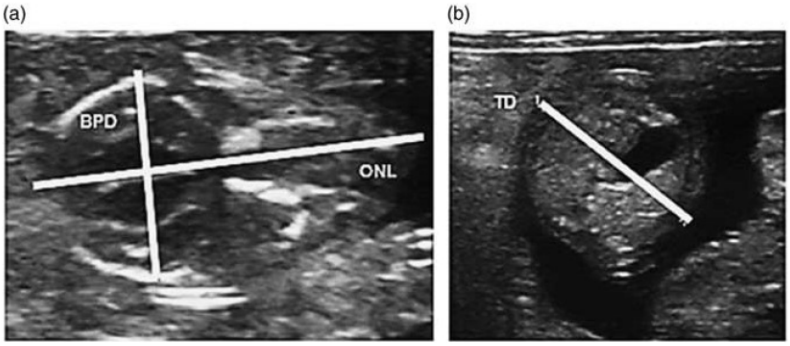

Fig. 1. Ultrasound image of the head (*a*) and the thorax (*b*) of a rabbit fetus at day 21 of pregnancy. ONL, occipito-nasal length; BPD, biparietal diameter; TD, thoracic diameter.

Morphometry of fetuses and offspring

Table 1 depicts the morphometric data of fetuses and kits. On day 21 of pregnancy maternal underfeeding was related to a reduction in fetal size, specifically in a shorter occipito-nasal length ($P < 0.05$), while biparietal and thoracic diameters were similar in control and underfed fetuses. At birth, a total of 43 newborns from control group and 53 in the underfed group were included in the study. Kits from the underfed group showed the lowest size (in terms of crown-rump length, biparietal and thoracic diameters; $P < 0.05$) and a decreased weight when compared with that of the control group ($P < 0.05$). Time of delivery was not affected by the feed restriction (all females delivered on day 31 of pregnancy). No difference was obtained between groups in the mortality rate at birth (1 out of 44 and 3 out of 56 in control and underfed groups, respectively; $P > 0.05$).

Doppler evaluation of fetoplacental hemodynamics

At the UCAs, significantly higher SPVs and time-averaged mean velocities ($P = 0.02$ and $P = 0.01$, respectively) were

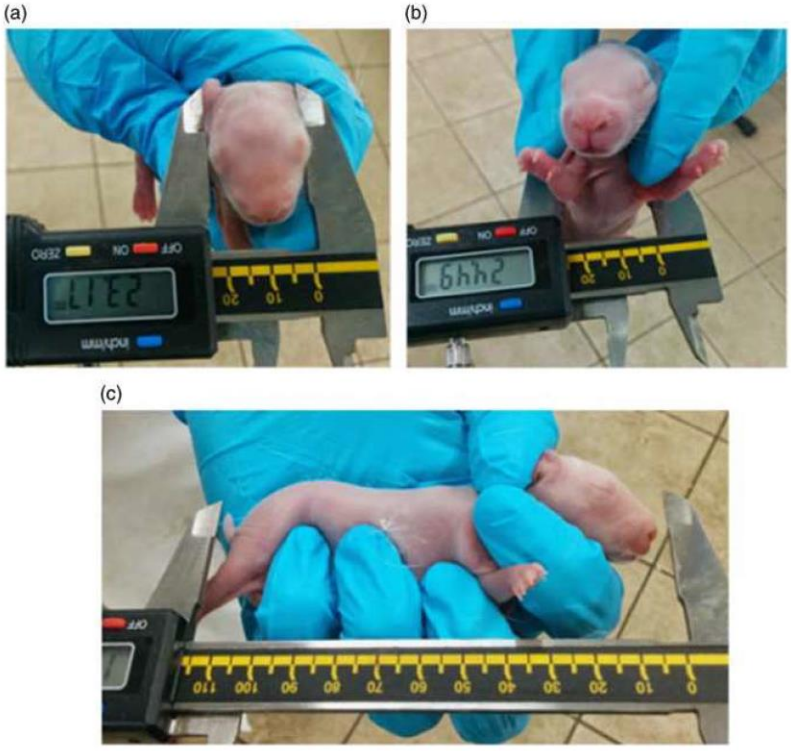

Fig. 2. Measurements obtained at birth: (*a*) biparietal diameter, (*b*) transversal thoracic diameter and (*c*) crown-rump length.

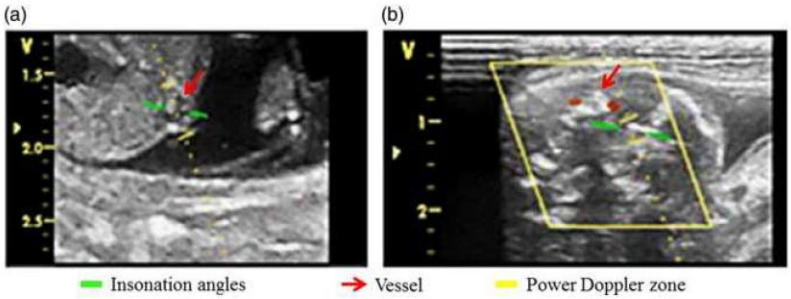

Fig. 3. Doppler image of UCA (*a*); and MCA (*b*) of a rabbit fetus at day 21 of pregnancy. UCA, umbilical cord artery; MCA, middle cerebral artery.

Table 1. *Offspring development of rabbit does in the control group and underfed group at day 21 of pregnancy and at birth*

	Control group	Underfed group
Fetal parameters	$n = 16$	$n = 20$
Occipito-nasal length (cm)	1.92 ± 0.07^{a}	1.60 ± 0.05^{b}
Biparietal diameter (cm)	1.02 ± 0.02	1.0 ± 0.02
Thoracic diameter (cm)	1.34 ± 0.04	1.32 ± 0.03
Kits parameters	$n = 43$	$n = 53$
Mortality rate (%)	2.43 (1/44)	5.35 (3/56)
Crown-rump length (cm)	10.75 ± 0.14^{a}	10.21 ± 0.12^{b}
Biparietal diameter (cm)	2.26 ± 0.02^{a}	2.03 ± 0.02^{b}
Thoracic diameter (cm)	2.37 ± 0.03^{a}	2.15 ± 0.03^{b}
Weight (g)	53.36 ± 1.70^{a}	48.0 ± 1.51^{b}

[a,b]Different superscripts within a row indicate significant differences among groups ($P < 0.05$).

observed in the underfed fetuses when compared with the control group (Table 2). Whereas, fetuses from the underfeed group showed a decreased EDV ($P = 0.05$). Indexes of pulsatility ($P = 0.07$) and resistance ($P = 0.06$) tended to be higher in the underfed fetuses, without being statistically significant.

There were no significant differences in the hemodynamic parameters at the MCA between both groups at any analyzed variable. Finally, the cerebroplacental ratio for MV was significantly lower in the underfed fetuses ($P = 0.02$).

Relationships between morphometric changes and hemodynamic parameters

Correlations between fetal size and hemodynamic features obtained by the Pearson procedure showed significant differences between the two nutritional regimes. In the control and underfed groups, the smallest fetuses, with smaller occipito-nasal length, had a lower EDV at UCA ($r = 0.671$, $P = 0.03$). There were no other significant effects in the control group; conversely, in the smallest fetuses of the underfed group, those affected by a more severe IUGR, a lower occipito-nasal length was also related to lower pulsatility and RIs ($r = 0.502$, $P = 0.03$; $r = 0.673$, $P = 0.02$, respectively), SPV and MV ($r = 0.763$ and $r = 0.698$, respectively; $P = 0.006$ for both) at the UCA. Moreover, restricted IUGR fetuses who had a smaller thoracic diameter showed increased cerebroplacental ratios for SPV and MV ($r = 0.524$, $P = 0.01$ and $r = 0674$, $P = 0.004$, respectively). Finally, a smaller biparietal diameter was related to an increased cerebroplacental ratio for EDV ($r = 0.394$, $P = 0.04$).

Discussion

The results of the present study show that underfeeding pregnant rabbits, at a level that does not affect maternal body weight, can be considered a useful model for IUGR studies. The level of restriction affected fetal development by reducing

Table 2. *Fetal hemodynamic parameters obtained from rabbit does in the control group and underfed group at day 21 of pregnancy*

	Control group ($n = 16$)	Underfed group ($n = 20$)
Resistance index		
UCA	0.79 ± 0.01	0.85 ± 0.01
MCA	0.71 ± 0.02	0.74 ± 0.02
Cerebroplacental ratio	0.90 ± 0.03	0.86 ± 0.03
Pulsatility index		
UCA	1.33 ± 0.04	1.42 ± 0.03
MCA	1.10 ± 0.04	1.20 ± 0.07
Cerebroplacental ratio	0.83 ± 0.04	0.84 ± 0.05
Systolic peak velocity		
UCA (cm/s)	26.71 ± 2.29^{a}	37.18 ± 3.33^{b}
MCA (cm/s)	18.41 ± 1.41	19.00 ± 1.19
Cerebroplacental ratio	0.70 ± 0.08	0.51 ± 0.05
End diastolic velocity		
UCA (cm/s)	5.40 ± 0.39^{a}	5.22 ± 0.35^{b}
MCA (cm/s)	5.39 ± 0.32	4.90 ± 0.41
Cerebroplacental ratio	1.07 ± 0.09	0.94 ± 0.09
Time-averaged mean velocity		
UCA (cm/s)	16.05 ± 1.26^{a}	22.40 ± 1.73^{b}
MCA (cm/s)	12.80 ± 0.99	11.60 ± 0.52
Cerebroplacental ratio	0.79 ± 0.09^{a}	0.53 ± 0.05^{b}

UCA, umbilical cord artery; MCA, middle cerebral artery.
[a,b]Different superscripts within a row indicate significant differences among groups ($P < 0.05$).

the prenatal growth of the offspring and therefore their size and weight at birth, without being associated to a higher mortality rate. Our data also indicate that IUGR fetuses develop compensative 'brain-sparing effect' for preserving adequate blood supply to the brain, with this effect being compromised in fetuses with severe IUGR. Thus, these data are similar to that previously reported in humans at 70% pregnancy.[26] Our study also confirms the adequacy of the use of ultrasound scanning for the assessment of fetal characteristics, reinforcing previous studies in rabbits.[20] This finding gives a way for future translational studies in this model since, in humans, ultrasound technology is widely used in prenatal care to estimate gestational age, to assess fetal growth and to determine physical abnormalities.[27] Moreover, fetal biometry during pregnancy is a confirmed method to predict probable later adverse perinatal outcomes.[28,29]

In the current study, ultrasonography showed fetal morphometric changes that were induced by maternal nutritional restriction at 70% pregnancy. Underfed fetuses showed a smaller occipito-nasal length than fetuses in the control group, without any other major changes. The occipito-nasal length is a more

accurate indirect measure for fetal development than other parameters since the skull remains in a good examinable position[30] and the hyperechogenic limit of the bones enables an easier measurement. From the morphometric changes, the limited impact of maternal restriction on embryo growth, together with the fact that rabbit and other species including human fetuses show a faster body growth toward the end of the pregnancy can be explained.[31] In the early pregnancy, embryo–fetal needs are scarce, with maternal metabolism being anabolic in this period[32] to ensure enough energetic reserves. Moreover, the does in our study were at optimal body weight at the beginning of the pregnancy and could have partially satisfied the low fetal growth requirements during the two first thirds of pregnancy without needing to mobilize their own body reserves.

The last third of pregnancy is characterized by maximum fetal growth rate and, therefore, due to the need for a higher amount of nutrients; maternal metabolism becomes catabolic,[33] and nutritional restriction during this phase of pregnancy normally has a severe impact on fetal growth, which caused the differences found at birth. In our study, feed intake was not significantly altered during the pregnancy in the control group, neither the maternal weight; this was consistent with other studies in rabbits with different levels of restriction showing no differences in maternal weight at the end of the trials.[34,35] Hence, we can hypothesize that despite the fetuses requiring a higher level of resources near pregnancy term, the mother may have invested her limited resources for her own metabolism, subsequent lactation and in her future reproduction instead of her litter growth or placental development.[36] There was not a higher mortality rate among restricted newborns, demonstrating that the maternal 'decision' of not dedicating more energy to their litter was right, in the view of survival rate. Probably a more severe restriction would induce changes in weight of the mothers, but further research is necessary to explore this issue and to determine if it would be a limiting factor in the translational value of the rabbit model.

The results obtained after the evaluation of the fetal blood flow by Doppler ultrasound in the present study reinforce the use of rabbits as a model for IUGR studies. Fetuses showed a high flow velocity at the umbilical arteries, resembling values in humans at the second trimester of pregnancy.[37–40] We focused our research on the umbilical artery since previous studies have shown that the measurement of insonation at umbilical arteries is easy to obtain and offers reliable data on blood exchange with the placenta than insonation of other vessels.[41] Hence, a high blood flow induces high PI and RI values, like in humans.[39]

In IUGR pregnancies, the increase in the resistance at the small arteries and arterioles of the villi, decreases the diastolic flow, which raises the MV at the UCAs.[40–42] Concomitantly, in the present study we observed that fetuses exposed to maternal underfeeding evidenced a lower EDV, higher systolic peak and time-averaged mean velocities, with a trend for higher indexes of pulsatility and resistance when compared with those of the control group. These alterations, usually detected in IUGR, are related to a lower supply of oxygen and nutrients to the fetus as a consequence of a placental ischemia.[42] Placental ischemia is considered the ultimate cause for IUGR and is associated with high perinatal morbility and mortality.[43] It could be hypothesized that fetuses in the underfed group were in the first stages of IUGR and demanded more blood flow to try to increase blood exchange rate,[43] since the nutrient content and oxygen supply were reduced. Consequently, the differences found in the current study could indicate a lower blood perfusion in fetuses from underfed mothers caused by the nutritional restriction.

Fetuses at IUGR risk, under circumstances such as poor nutrition or inadequate placental supply, develop a redistribution of blood circulation to increase the blood supply to the fetal brain. This phenomenon is known as 'the compensatory brain-sparing effect'[44] and its purpose is to assure adequate brain development and function, since a failure of this causes a broad spectrum of adverse neurological outcomes, thus compromising postnatal vitality and survival of the neonate.[45,46] In human medicine, the occurrence of brain sparing consecutive to IUGR has been identified by assessing Doppler ratios.[44] The cerebroplacental ratios quantify the redistribution of the cardiac output by dividing Doppler index from cerebral and fetoplacental vascularity. In this scenario, umbilical arteries represent the right ventricular afterload, whereas, the MCA reflects the left ventricular afterload.[47]

The brain-sparing effect was manifest in the underfed fetuses of the current study (significantly lower cerebroplacental ratio and a trend for higher PI); this is evidence of a redistribution of the cardiac output to the cerebral circulation (Table 2). Our study also identified the weakness and even the failure of this compensatory mechanism in those fetuses suffering a more severe IUGR, those with smaller biparietal and thoracic diameters, as evidenced by the Pearson correlations.

Our results indicate the reliability and usefulness of the rabbit model and of the Doppler procedure for the study of IUGR. However, we cannot leave aside some limitations of this study. The first limitation would be a systematic assessment of the changes in IUGRs throughout the entire pregnancy. However, we preferred limited scanning sessions since repeated examinations of the pregnant does could have caused stress-induced abortion, even in trained animals, especially during the last third of pregnancy, when the decidua, the maternal side of the placenta is thinner and less attached.[48] A second limitation would be the small sample size used in this study, which could have reduced the power of the study and masked significant differences. Finally, more information about the UCA diameters could have been relevant, there can be errors when measuring vessel diameter, in particular for vessels with a high pulsating blood flow.[49] In our case those measurements were more complicated since no anesthetic drugs were used, since they could interference with maternal uteroplacental blood flow.

In conclusion, our data indicate that rabbit fetuses exposed to maternal underfeeding are prone to show a disrupted developmental trajectory and, due to this restriction, to

undergo IUGR. IUGR fetuses were able to develop compensative brain-sparing effects in a similar way to that previously described for humans. Thus, this study supports the rabbit as a reliable translational model for studies on IUGR associated with feed restriction and associated diseases. The use of the Doppler technique to explore umbilical arteries, as well as the estimation of the cerebroplacental ratios led to the early detection of fetal blood flow alterations and should be taken in consideration as a primary diagnosis tool. The information herein could be of interest to researchers studying the vascular adaptation and/or deterioration processes and help in exploring possible therapies in experimental models of IUGR.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (European Union Directive about the protection of animals used in experimentation and the Spanish policy for animal protection RD53/2013) and has been approved by the institutional committee (Polytechnic University of Madrid), which meets the requirements of the European Union for scientific procedure establishment, under project license of the UPM Scientific Ethic Committee.

References

1. Brett KE, Ferraro ZM, Yockell-Lelievre J, Gruslin A, Adamo KB. Maternal–fetal nutrient transport in pregnancy pathologies: the role of the placenta. *Int J Mol Sci.* 2014; 15, 16153–16185.
2. Ramakrishnan U, Imhoff-Kunsch B, Martorell R. Maternal nutrition interventions to improve maternal, newborn, and child health outcomes. *Nestle Nutr Inst Workshop Ser.* 2014; 78, 71–80.
3. Murthy LS, Agarwal KN, Khanna S. Placental morphometric and morphologic alterations in maternal undernutrition. *Am J Obstet Gynecol.* 1976; 124, 641–646.
4. Chia CC, Huang SC. Overview of fetal growth retardation/restriction. *Taiwan J Obstet Gynecol.* 2014; 3, 435–440.
5. Bhutta ZA, Das JK, Rizvi A, *et al.* Evidence-based interventions for improvement of maternal and child nutrition: what can be done and at what cost? *Lancet.* 2013; 382, 452–477.
6. Cetin I, Mando C, Calabrese S. Maternal predictors of intrauterine growth restriction. *Curr Opin Clin Nutr Metab Care.* 2013; 16, 310–319.
7. Nardozza LM, Araujo Junior E, Barbosa MM, *et al.* Fetal growth restriction: current knowledge to the general Obs/Gyn. *Arch Gynecol Obstet.* 2012; 286, 1–13.
8. Ergaz Z, Avgil M, Ornoy A. Intrauterine growth restriction-etiology and consequences: what do we know about the human situation and experimental animal models? *Reprod Toxicol.* 2005; 20, 301–322.
9. Redmer DA, Wallace JM, Reynolds LP. Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domest Anim Endocrinol.* 2004; 27, 199–217.
10. Gonzalez-Bulnes A, Ovilo C, Lopez-Bote CJ, *et al.* Fetal and early-postnatal developmental patterns of obese-genotype piglets exposed to prenatal programming by maternal over- and undernutrition. *Endocr Metab Immune Disord Drug Targets.* 2013; 13, 240–249.
11. Ross MG, Desai M. Developmental programming of offspring obesity, adipogenesis, and appetite. *Clin Obstet Gynecol.* 2013; 56, 529–536.
12. Barker DJ. In utero programming of cardiovascular disease. *Theriogenology.* 2000; 53, 555–574.
13. Saffery R. Epigenetic change as the major mediator of fetal programming in humans: are we there yet? *Ann Nutr Metab.* 2014; 64, 203–207.
14. Schroder HJ. Models of fetal growth restriction. *Eur J Obstet Gynecol Reprod Biol.* 2003; 110, S29–S39.
15. Puschel B, Daniel N, Bitzer E, *et al.* The rabbit (Oryctolagus cuniculus): a model for mammalian reproduction and early embryology. *Cold Spring Harb Protoc.* 2010; 2010, pdb.emo139.
16. Fischer B, Chavatte-Palmer P, Viebahn C, Santos AN, Duranthon V. Rabbit as a reproductive model for human health. *Reproduction.* 2012; 144, 1–10.
17. Russell WMS, Burch RL, Hume CW. *The Principles of Humane Experimental Technique.* 1959. Universities Federation for Animal Welfare: Wheathampstead, England.
18. Derrick M, Drobyshevsky A, Ji X, *et al.* Hypoxia–ischemia causes persistent movement deficits in a perinatal rabbit model of cerebral palsy: assessed by a new swim test. *Int J Dev Neurosci.* 2009; 27, 549–557.
19. van Vliet E, Eixarch E, Illa M, *et al.* Metabolomics reveals metabolic alterations by intrauterine growth restriction in the fetal rabbit brain. *PLoS One.* 2013; 8, e64545.
20. Chavatte-Palmer P, Laigre P, Simonoff E, *et al.* In utero characterisation of fetal growth by ultrasound scanning in the rabbit. *Theriogenology.* 2008; 69, 859–869.
21. Huizinga CT, Engelbregt MJ, Rekers-Mombarg LT, *et al.* Ligation of the uterine artery and early postnatal food restriction – animal models for growth retardation. *Horm Res.* 2004; 62, 233–240.

22. Eixarch E, Hernandez-Andrade E, Crispi F, *et al.* Impact on fetal mortality and cardiovascular Doppler of selective ligature of uteroplacental vessels compared with undernutrition in a rabbit model of intrauterine growth restriction. *Placenta.* 2011; 32, 304–309.
23. Rebollar PG, Dal Bosco A, Millán P, *et al.* Ovulating induction methods in rabbit does: the pituitary and ovarian responses. *Theriogenology.* 2012; 77, 292–298.
24. Hoffman LH, Breinan DR, Blaeuer GL. The rabbit as a model for implantation: in vivo and in vitro studies. In *Embryo Implantation: Molecular, Cellular, and Clinical Aspects* (ed. Carson DD), 1999; pp. 151–160. Springer-Verlag: New York, NY.
25. Hafez ES, Tsutsumi Y. Changes in endometrial vascularity during implantation and pregnancy in the rabbit. *Am J Anat.* 1966; 118, 249–282.
26. Vik T, Vatten L, Jacobsen G, Bakketeig LS. Prenatal growth in symmetric and asymmetric small-for-gestational-age infants. *Early Hum Dev.* 1997; 48, 167–176.
27. Smarr MM, Vadillo-Ortega F, Castillo-Castrejon M, O'Neill MS. The use of ultrasound measurements in environmental epidemiological studies of air pollution and fetal growth. *Curr Opin Pediatr.* 2013; 25, 240–246.
28. Smith GC, Smith MF, McNay MB, Fleming JE. First-trimester growth and the risk of low birth weight. *N Engl J Med.* 1998; 339, 1817–1822.
29. Pedersen NG, Wojdemann KR, Scheike T, Tabor A. Fetal growth between the first and second trimesters and the risk of adverse pregnancy outcome. *Ultrasound Obstet Gynecol.* 2008; 32, 147–154.
30. Kelly RW, Newnham JP. Estimation of gestational age in Merino ewes by ultrasound measurement of fetal head size. *Aust J Agri Res.* 1989; 40, 1293–1299.
31. Beaudoin S, Barbet P, Bargy F. Developmental stages in the rabbit embryo: guidelines to choose an appropriate experimental model. *Fetal Diagn Ther.* 2003; 18, 422–427.
32. Vernon RG, Clegg RA, Flint DJ. Adaptations of adipose tissue metabolism and number of insulin receptors in pregnant sheep. *Comp Biochem Physiol B.* 1985; 81, 909–913.
33. Symonds ME, Clarke L. Nutrition-environment interactions in pregnancy. *Nutr Res Rev.* 1996; 9, 135–148.
34. Nafeaa A, Ahmed SA, Fat Hallah S. Effect of feed restriction during pregnancy on performance and productivity of New Zealand white rabbit does. *Vet Med Int.* 2011; 2011, 839737.
35. Menchetti L, Brecchia G, Cardinali R, Polisca A, Boiti C. Food restriction during pregnancy: effects on body condition and productive performance of primiparous rabbit does. *World Rabbit Sci.* 2015; 23, 1–8.
36. Fowden AL, Moore T. Maternal-fetal resource allocation: co-operation and conflict. *Placenta.* 2012; 33, 11–15.
37. Ichizuka K, Ando S, Ichihara M. Application of high-intensity focused ultrasound for umbilical artery occlusion in a rabbit model. *Ultrasound Obstet Gynecol.* 2007; 30, 47–51.
38. Hershkovitz R, Kingdom JCP, Geary M, Rodeck CH. Fetal cerebral blood flow redistribution in late gestation: identification of compromise in small fetuses with normal umbilical artery Doppler. *Ultrasound Obstet Gynecol.* 2000; 15, 209–212.
39. Polisca A, Scotti L, Orlandi R, Brecchia G, Boiti C. Doppler evaluation of maternal and fetal vessels during normal gestation in rabbits. *Theriogenology.* 2010; 73, 358–366.
40. Gudmundsson S, Marsal K. Umbilical artery and uteroplacental blood flow velocity waveforms in normal pregnancy – a crosssectional study. *Eur J Obstet Gynaecol.* 1988; 67, 347–354.
41. Acharya G, Wilsgaard T, Berntsen GK, Maltau JM, Kiserud T. Reference ranges for serial measurements of blood velocity and pulsatility index at the intra-abdominal portion, and fetal and placental ends of the umbilical artery. *Ultrasound Obstet Gynecol.* 2005; 26, 162–169.
42. Bamfo JE, Odibo AO. Diagnosis and management of fetal growth restriction. *J Pregnancy.* 2011; 2011, 640715.
43. Roberts JM. Pathophysiology of ischemic placental disease. *Semin Perinat.* 2014; 38, 139–145.
44. Makhseed M, Jirous J, Ahmed MA, Viswanathan DL. Middle cerebral artery to umbilical artery resistance index ratio in the prediction of neonatal outcome. *Int J Gynaecol Obstet.* 2000; 71, 119–125.
45. Peeling AN, Smart JL. Review of literature showing that undernutrition affects the growth rate of all processes in the brain to the same extent. *Metab Brain Dis.* 1994; 9, 33–42.
46. Salihagic-Kadic A, Medic M, Jugovic D, *et al.* Fetal cerebrovascular response to chronic hypoxia – implications for the prevention of brain damage. *J Matern Fetal Neonatal Med.* 2006; 19, 387–396.
47. Baschat AA, Gembruch U. The cerebroplacental Doppler ratio revisited. *Ultrasound Obstet Gynecol.* 2003; 21, 124–127.
48. Mossman HW. The rabbit placenta and the problem of placental transmission. *Am J Anat.* 1926; 37, 433–497.
49. Hecher K, Campbell S, Doyle P, Harrington K, Nicolaides K. Assessment of fetal compromise by Doppler ultrasound investigation of the fetal circulation arterial, intracardiac, and venous blood flow velocity studies. *Circulation.* 1995; 91, 129–138.

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The effects of sildenafil citrate on feto–placental development and haemodynamics in a rabbit model of intrauterine growth restriction

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Abstract. The present study evaluated the effectiveness of sildenafil citrate (SC) to improve placental and fetal growth in a diet-induced rabbit model of intrauterine growth restriction (IUGR). Pregnant rabbits were fed either *ad libitum* (Group C) or restricted to 50% of dietary requirements (Group R) or restricted and treated with SC (Group SC). The treatment with SC improved placental development by increasing vascularity and vessel hypertrophy in the decidua. The assessment of feto–placental haemodynamics showed higher resistance and pulsatility indices at the middle cerebral artery (MCA) in fetuses treated with SC when compared with Group R, which had increased systolic peak and time-averaged mean velocities at the MCA. Furthermore, fetuses in the SC group had significantly higher biparietal and thoracic diameters and longer crown–rump lengths than fetuses in Group R. Hence, the SC group had a reduced IUGR rate and a higher kit size at birth compared with Group R. In conclusion, SC may provide potential benefits in pregnancies with placental insufficiency and IUGR, partially counteracting the negative effects of food restriction on placental development and fetal growth. However, the present study also found evidence of a possible blood overflow in the brain that warrants further investigation.

Additional keywords: fetus, placenta, pregnancy.

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Introduction

The failure of fetuses to achieve their full growth potential is known as intrauterine growth restriction (IUGR). Currently, between 5 and 10% of human infants undergo IUGR (Nardozza *et al.* 2012) and, as a consequence, are at greater risk of neonatal health disorders (Maršál 2002) and late-onset diseases in adulthood (Ross and Desai 2013). The aetiology of IUGR is multifactorial and scarcely understood, but is thought to include a combination of maternal, environmental, fetal and placental factors negatively affecting fetal homeostasis (Sankaran and Kyle 2009).

The intrauterine environmental conditions of the fetus are regulated by the placenta. At present, more than 60% of IUGR offspring in developed countries are linked to abnormal placental development or placental insufficiency (Ghidini 1996). Thus, research has been focussed on devising preventive and therapeutic strategies for IUGR and, specifically, on developing therapies to improve placental development and utero–placental blood flow. An encouraging area of research is the stimulation of the placental pro-angiogenic factors placental growth factor (PlGF) and vascular endothelial growth factor (VEGF), which are primarily driven by nitric oxide (NO) and its endothelial

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constitutive synthase (eNOS or NOS3). NO is a potent stimulator of vasodilatation and angiogenesis during placental development (Purcell *et al.* 1999) and decreased NO bioavailability is recognised to be involved in the pathogenesis of IUGR (Serrano *et al.* 2004). Based on this, a possible therapeutic strategy would be the administration of sildenafil citrate (SC), a vasodilator molecule that enhances NO concentrations by inhibiting phosphodiesterase-5 (PDE-5) activity (Chuang *et al.* 1998), which may optimise placental function and, therefore, alleviate IUGR in at-risk pregnancies. After preclinical studies, SC is being tested in women with promising results, improving maternal and fetal blood flow velocimetry and fetal well being (Lacassie *et al.* 2004; Lin *et al.* 2012; Panda *et al.* 2014; Sun *et al.* 2014; Trapani *et al.* 2015). Currently, several clinical trials are underway to further test the usefulness and safety of SC treatments for IUGR (Ganzevoort *et al.* 2014).

Research in human pregnancies is obviously limited by ethical and practical limitations and necessitates the use of animal models. Most studies of IUGR have been performed in rodent models (Schroder 2003). However, the rabbit is an emergent and complementary model for pregnancy studies (Eixarch *et al.* 2009; Püschel *et al.* 2010). The size of a rabbit allows serial blood sampling and imaging and also shows more similarities in metabolic, endocrine, placental and fetal features to humans than rodents (Kobayashi *et al.* 2011; Fischer *et al.* 2012; Malassiné *et al.* 2013). Specifically, the rabbit placenta is haemodichorial, which is more physiologically similar to the haemomonochorial human placenta than the haemotrichorial placenta of rodents (Fischer *et al.* 2012). Haemodynamic changes in the placenta during pregnancy in rabbits are also comparable to those in humans (Fischer *et al.* 2012; Lecarpentier *et al.* 2012), with high blood flow velocities in the umbilical arteries resembling human values in the second trimester (Polisca *et al.* 2010). Additionally, brain white-matter maturation in rabbits occurs during the perinatal period, similarly to humans, whilst in rodents this process occurs largely in the postnatal period (Beaudoin *et al.* 2003; Derrick *et al.* 2009).

Different studies in rodent models have demonstrated that SC administration during pregnancy prevents the production of inflammatory cytokines, prevents fetal loss (Luna *et al.* 2015), improves feto–placental blood flow (Stanley *et al.* 2012) and increases fetal weight (Stanley *et al.* 2012; Dilworth *et al.* 2013). However, most of the results were obtained post mortem and the ontogeny of changes in feto–placental haemodynamics and intrauterine growth are unknown.

Hence, the present study evaluated whether maternal SC administration could improve or ameliorate diet-induced defects in feto–placental development, haemodynamics and offspring outcome seen in rabbits exposed to 50% food restriction from Day 9 of pregnancy onwards, a model previously developed in our laboratory (López-Tello *et al.* 2015).

Materials and methods

Ethical approval

All experiments were carried out at the animal facilities of the Polytechnic University of Madrid (UPM, Spain), which meet the requirements of the European Union for scientific procedure establishments, under project licence of the UPM Scientific Ethic Committee. Animal manipulations were performed in accordance with the Spanish policy for animal protection RD53/2013, which complied with the European Union Directive about the protection of animals used in experimentation.

Animals and management

The experiment involved 45 New Zealand × California White rabbits (*Oryctolagus cuniculus*). Females were previously artificially inseminated and during the trial the animals were kept in individual cages under a constant photoperiod of 16 h light per day. A temperature of 18–22°C and a relative humidity of 60–75% were maintained by a forced ventilation system, according to the normal husbandry conditions for rabbits (Rebollar *et al.* 2012). All females had free access to water and were fed a diet containing 16% crude protein, 37% crude fibre, 3.7% fat and crude energy content of 2400 kcal kg^{-1} (Nanta, Spain). Daily food intake of dams was determined individually (2 weeks before starting the experiment). Food intake was measured daily by weighing the food and feeder at the beginning and at the end of the adjustment period. The mean food intake of all dams was 187.0 ± 11.0 g day^{-1}.

Experimental design

At Day 9 of pregnancy (term = Day 31), females were randomly distributed into three experimental groups. The first group was fed *ad libitum* during the entire pregnancy and considered to be the control group (Group C; $n = 15$), whilst the remaining dams were restricted individually to 50% of their average daily food intake until parturition. From Day 22 of pregnancy to delivery, half of the restricted dams were treated daily with oral SC. This was prepared by grinding Viagra tablets (100 mg; Pfizer, USA; 5 mg kg^{-1} excluding excipients) and mixing in 1 mL of baby food (Hero Baby, Spain; Group SC, $n = 15$). The remaining restricted dams received no other treatment and were considered as the untreated controls of food restriction (Group R, $n = 15$). Day 22 was chosen because it corresponds to the beginning of the period in pregnancy in which enlargement of the uterus ceases, the somatic circulation rate decreases and the incidence of IUGR is augmented due to the increase in the requirements of the fetuses for oxygen and nutrients (Reynolds 1946; López-Tello *et al.* 2015). The SC dose used in this trial was adjusted according to previous studies performed in rabbits and rats (Park *et al.* 2004; Cauli *et al.* 2010) and it was administrated orally with a syringe once per day (0900 hours) to ensure that each animal received the adequate dose, avoiding any under- or over-dosing. The use of a unique dose per day was based on the protocols of Sánchez-Aparicio *et al.* (2008) in guinea pigs and guidelines from the FDA Center for Drug Evaluation and Research in pregnant rabbits (http://www.accessdata.fda.gov/drugsatfda_docs/NDA/98/viagra/pharm_tox_pp_117_114.pdf, verified 28 April 2016). Both control groups (C and R) also received 1 mL of baby food at the same time as treated dams to avoid any possible confounding effect.

Four days after SC administration (Day 26 of pregnancy; ≈84% of the total pregnancy), four females of each group were randomly submitted to a Doppler evaluation. At Day 28 of

pregnancy (≈90% of the total pregnancy), 22 dams were killed to study feto–placental morphology and the remaining females were allowed to deliver, registering data from newborns.

Study of fetuses and placentas

The dams were sedated with 35 mg kg^{-1} ketamine (Imalgene1000; Merial, Spain) and then killed using an intravenous bolus of barbiturate (30 mg kg^{-1}; Dolethal; Vetoquinol, Spain). A mid-ventral abdominal laparotomy was made to remove the entire reproductive tract. Fetuses were dissected from their extra-embryonic membranes and considered as either: (1) viable fetus (presented natural morphological features according to age and bodyweight; see Fig. S1*a*, available as Supplementary Material to this paper), (2) mummified or dead fetus (excluded from trial as we could not determinate the exact time of death; Fig. S1*b*) or (3) resorption (with atrophied fetal and maternal placenta; Fig. S1*c*).

For the viable fetuses, placentas were immediately and gently separated from the decidua (attached to the endometrium and comprised of uninucleated and giant cells in a matrix of collagen in which maternal blood passes to the implantation site through spiral arteries; Samuel *et al.* 1975) and the labyrinth (mainly composed of fetal and maternal capillaries and trophoblast responsible for nutrient and oxygen exchange; Fig. S1*d*). Both compartments were weighed and the length and thickness measured using slide calipers (values were obtained by considering the average of three consecutive measurements). Following this, fetuses were weighed and measured for crown–rump length (CRL, maximum distance from crown to tail), biparietal diameter (BPD, length from one parietal eminence to the other) and transversal thoracic diameter (TD, length at the diaphragm insertion). Fetuses were beheaded at the atlanto–occipital joint and, after cranial opening and medial laparotomy, fetal brain and liver were removed and weighed.

A viable fetus was considered to have been exposed to IUGR when its bodyweight was below the 10th percentile, assuming the control group as our standard value. Afterwards, different ratios were obtained by dividing fetal (head, brain and liver) and placental structures (decidua and labyrinth zones) by fetal weight. The weight of the brain relative to the liver was also considered as an indicator of IUGR. Finally, in order to evaluate fetal metabolic status, a total of 30 fetuses from each group were randomly selected. Blood samples were obtained after decapitation and placed in tubes with ethylenediamine tetraacetic acid (EDTA), centrifuged at 1200*g* for 10 min at 4°C to obtain plasma and immediately stored at −20°C until analysis. Parameters related to the metabolism of glucose and lipids (triglycerides and cholesterol) were measured with a clinical chemistry analyser (Saturno 300 plus; Crony Instruments, Italy) according to the manufacturer's instructions.

Placental histopathology

Sections of placentas and uteri adjacent to the ovary were collected ($n = 10$ per group), fixed in 4% paraformaldehyde for 24 h and switched to 70% ethanol for histological evaluation. Samples were embedded in paraffin, sectioned at 4-μm thickness and stained with haematoxylin–eosin following routine laboratory procedures. Sections were examined histologically by a trained pathologist blinded for the experimental procedure.

Feto–placental haemodynamics

Ultrasound scanning was carried out with a Vivid-I ultrasound machine equipped with a multi-frequency (8–12 MHz) linear array probe (General Electrics Ultraschall Deutschland GmbH, Germany). In brief, fasted animals were shaved at the abdominal area and gently restrained in dorsal recumbence, without anaesthesia to avoid any effect on heart rate and blood flow during the observations. Females usually stayed calm and relaxed during the procedure since they were regularly handled by research staff. A complete scan of the dam did not last more than 20 min. Measurements were taken from 48 fetuses (four fetuses from each female in order to minimise individual effects).

Blood-flow parameters of umbilical cord arteries (UCA) and middle cerebral arteries (MCA) were determined after identifying the vessels with colour Doppler. The waveforms of three consecutive cardiac cycles in each vessel were recorded, disregarding views with angles of insonation between 20 and 60°. Measurements were obtained after the entire examination, recording and including resistance index (RI), pulsatility index (PI), systolic peak velocity (SPV), end diastolic velocity (EDV) and time-averaged mean velocity (MV), measured at both UCA and MCA.

Neonatal study

Twenty-three dams were allowed to deliver in order to study the effects of food restriction and SC administration during pregnancy on the neonates. Immediately after birth, all the kits ($n = 251$) were classified as viable newborns ($n = 236$) or stillborns ($n = 15$). Bodyweight and morphometric parameters (CRL, BPD and TD) were measured only in viable newborns. A newborn was considered to have IUGR when its bodyweight was below the 10th percentile assuming the control group at birth as our standard value.

Statistical analysis

Statistical analyses were performed with Statistical Analysis System Software (SAS Institute Inc. Cary, NC, USA). Effects of undernutrition and sildenafil treatment on the morphological parameters of fetuses, placentas and newborns and the haemodynamic parameters of fetuses were assessed by one-way analysis of variance (one-way ANOVA); *t*-test was performed to contrast the differences between groups. The number of fetuses or kits per dam was used as a covariate. Possible differences in IUGR rate and number of placentas with histological findings were calculated by a χ^2 test. All data are reported as mean ± s.e.m. and probabilities were considered to be significant at $P < 0.05$.

Results

Morphological study of fetuses and placentas

The number of total (C, 11.95 ± 0.77; R, 12.70 ± 0.70; SC, 12.75 ± 0.75), viable (C, 11.55 ± 0.78; R, 11.60 ± 0.71; SC, 11.59 ± 0.76) and mummified (C, 0.15 ± 0.14; R, 0.22 ± 0.19; SC, 0.13 ± 0.13) fetuses and resorptions (C, 0.25 ± 0.20;

Table 1. Morphometric study of fetuses at Day 28 of pregnancy from dams fed *ad libitum* (C), restricted diet (R) or restricted diet and treated with sildenafil citrate (SC)

Statistical analyses were performed by one-way ANOVA and *t*-test mean comparison test. IUGR rate defined as those fetuses under 10th percentile of *ad libitum* control weights (32 g) estimated by a χ^2 test. Data represented as mean ± s.e.m. [a,b,c]Different superscripts within a row indicate significant differences between groups; $P < 0.05$

Parameter	C ($n = 81$)	R ($n = 94$)	SC ($n = 77$)	$P >$ f
Morphometric measurements				
Biparietal diameter (cm)	1.76 ± 0.02^a	1.67 ± 0.02^b	1.82 ± 0.02^c	0.001
Crown–rump length (cm)	10.52 ± 0.08^a	9.95 ± 0.07^b	10.32 ± 0.08^a	0.001
Thoracic diameter (cm)	1.82 ± 0.02^a	1.63 ± 0.02^b	1.70 ± 0.02^c	0.001
Fetus weights				
Total (g)	39.28 ± 0.64^a	33.91 ± 0.59^b	34.30 ± 0.66^b	0.001
Head (g)	9.46 ± 0.15^a	8.47 ± 0.14^b	8.67 ± 0.16^b	0.001
Body (g)	29.50 ± 0.52^a	25.34 ± 0.49^b	25.44 ± 0.54^b	0.001
Liver (g)	2.90 ± 0.07^a	2.52 ± 0.07^b	2.63 ± 0.08^b	0.001
Brain (g)	1.05 ± 0.01^a	0.99 ± 0.01^b	1.00 ± 0.01^b	0.001
Weight ratios				
Head weight (%)	24.22 ± 0.32^a	25.36 ± 0.30^b	25.40 ± 0.33^b	0.015
Brain ratio (%)	2.74 ± 0.05^a	3.05 ± 0.04^b	2.94 ± 0.05^b	0.001
Liver ratio (%)	7.34 ± 0.14^a	7.32 ± 0.13^a	7.74 ± 0.14^b	0.035
Brain : liver ratio (%)	38.66 ± 1.16^a	43.65 ± 1.09^b	39.01 ± 1.19^a	0.002
IUGR rate (%)	9.87^a	44.68^b	29.87^c	0.001

R, 0.27 ± 0.19; SC, 0.27 ± 0.20) were similar among groups. All the values for morphometric measurements were lower in Group R than in Group C ($P < 0.05$; Table 1). Conversely, the BPD of the SC group was higher than in Groups C and R ($P < 0.05$), whilst the CRL was similar to Group C and greater than Group R. For the SC group, the TD was lower than Group C but greater than Group R. Food restriction (Groups R and SC) reduced the weight of fetuses as well as the weight of head, body, liver and brain when compared with Group C ($P < 0.05$). However, SC administration was associated with an intermediate value of IUGR rate when compared with Groups C and R (Table 1 and Fig. S2). The ratios of head and brain to fetal weight (Table 1) were significantly higher in Groups R and SC ($P < 0.05$), whilst the brain to liver weight ratio was only higher in Group R ($P < 0.05$). In contrast, the ratio of liver to fetal weight was higher only in Group SC ($P < 0.05$).

With regards to metabolic status, fetuses from Group SC presented higher plasma glucose concentrations compared with Groups C and R (104.23 ± 6.72 vs 76.91 ± 8.49 and 79.35 ± 6.84 mg dL^{-1}, respectively; $P < 0.05$). However, no differences in plasma cholesterol (C, 85.17 ± 4.22; R, 81.84 ± 3.32; SC, 84.64 ± 3.28 mg dL^{-1}) or triglyceride concentrations (C, 116.90 ± 7.05; R, 116.84 ± 5.70; SC, 112.01 ± 5.87 mg dL^{-1}) were found among groups.

Maternal food restriction was also found to be related to changes in placental development in both the decidua and the labyrinth compartment (Table 2). The decidua was significantly thinner in Group R, whereas placentas in the group treated with SC had similar values to Group C. On the other hand, there was a trend for a thicker labyrinth in Group SC than in Group R ($P = 0.08$). The food restriction in both SC and R groups reduced the length of decidua and labyrinth, but did not affect compartment weight. Finally, the weight of the placenta relative to fetal weight, total placental ratio, was significantly higher in Group SC than in Group C ($P < 0.05$). The ratio between the decidua and fetal weight for Group SC showed intermediate values between Groups C and R, whilst the labyrinth to fetal weight ratio was significantly higher in Group SC when compared with the other two groups ($P < 0.05$).

Placental histopathology

Significant histological findings are summarised in Table 3 and Fig. 1. Histological changes in the placental structure were found in the junction zone and decidua region of both restricted groups (R and SC). The junction zones of a high percentage of placentas from Group R contained moderately increased amounts of poorly cellular fibrous connective tissue that extended multi-focally into the labyrinth and surrounds, and replaced vascular channels with the collapse of the adjacent labyrinth structure. Additionally, the decidua from the animals of Group R was moderately thinned when compared with the C and SC groups. On the other hand, there was a higher percentage of labyrinth and decidua samples containing moderately to markedly increased numbers of dilated small capillaries, venules and arterioles in the SC group. Interestingly, these placentas presented a higher number of and more dilated arterial sinuses compared with placentas from control and restricted animals without treatment.

Feto–placental haemodynamics

The results obtained at Day 26 of pregnancy showed a trend for higher RI ($P = 0.06$) and a significantly higher SPV ($P < 0.05$) in the UCA in both restricted groups with respect to fetuses from Group C (Table 4). There were significant effects of SC treatment on the blood flow in the fetal MCA, with fetuses in

Table 2. Placental dimensions obtained at Day 28 of pregnancy in dams fed *ad libitum* (C), restricted diet (R) or restricted diet and treated with sildenafil citrate (SC)

Statistical analyses were performed by one-way ANOVA and *t*-test mean comparison test. Data represented as mean ± s.e.m. IUGR rate estimated by a χ^2 test. [a,b]Different superscripts within a row indicate significant differences between groups; $P < 0.05$

Parameter	C ($n = 81$)	R ($n = 94$)	SC ($n = 77$)	$P >$ f
Total placental weight (g)	5.62 ± 0.16	5.28 ± 0.15	5.27 ± 0.16	0.205
Total placental weight/fetal weight (%)	14.74 ± 0.41^{a}	15.57 ± 0.37^{ab}	16.01 ± 0.40^{b}	0.030
Decidual zone				
Weight (g)	1.28 ± 0.04	1.24 ± 0.04	1.15 ± 0.04	0.100
Length (cm)	3.65 ± 0.09^{a}	3.23 ± 0.09^{b}	3.36 ± 0.10^{b}	0.006
Thickness (cm)	0.32 ± 0.02^{a}	0.26 ± 0.02^{b}	0.35 ± 0.02^{a}	0.001
Decidua weight/fetal weight (%)	3.41 ± 0.16^{a}	3.88 ± 0.15^{b}	3.49 ± 0.16^{a}	0.038
Labyrinth zone				
Weight (g)	3.93 ± 0.14	3.64 ± 0.13	3.8 ± 0.14	0.323
Length (cm)	3.61 ± 0.06^{a}	3.27 ± 0.05^{b}	3.34 ± 0.06^{b}	0.001
Thickness (cm)	0.47 ± 0.02	0.43 ± 0.01	0.48 ± 0.02	0.084
Labyrinth weight/fetal weight (%)	10.27 ± 0.34^{a}	10.62 ± 0.31^{a}	11.53 ± 0.34^{b}	0.029

Table 3. Characteristic placental histology at Day 28 of pregnancy in dams fed *ad libitum* (C), restricted diet (R) or restricted diet treated with sildenafil citrate (SC)

Statistical analyses were performed by χ^2 test. Data represented as mean ± s.e.m. [a,b]Different superscripts within a row indicate significant differences between groups; $P < 0.05$ (number of placentas with findings compared with the total number of samples)

Parameter	C ($n = 10$)	R ($n = 10$)	SC ($n = 10$)	$P >$ f
Labyrinth zone				
Collapse and fibrosis (%)	10^{a} (1/10)	50^{b} (5/10)	0^{a} (0/10)	0.009
Junctional zone				
Fibrosis (%)	10^{a} (1/10)	60^{b} (6/10)	0^{a} (0/10)	0.001
Increased vascularity (%)	0^{a} (0/10)	0^{a} (0/10)	80^{b} (8/10)	0.001
Decidual zone				
Atrophy (%)	0^{a} (0/10)	70^{b} (7/10)	0^{a} (0/10)	0.001
Hyperplastic arterial sinuses (%)	0^{a} (0/10)	0^{a} (0/10)	80^{b} (8/10)	0.001

Group SC showing higher RI and PI than those from the other two groups ($P < 0.05$). SPV and MV measurements were also higher in the SC group than for fetuses in Group C ($P < 0.05$).

Neonatal morphological study

At parturition, no significant differences in the total number of kits delivered (C, 11.62 ± 0.88; R, 11.28 ± 1.01; SC, 9.72 ± 1.37), newborns (C, 10.62 ± 0.84; R, 10.71 ± 0.92; SC, 9.28 ± 1.25) or stillborns (C, 1 ± 0.62; R, 0.57 ± 0.30; SC, 0.42 ± 0.30) were found. Group C had the highest values for average bodyweight and morphometric measurements of BPD, CRL and TD ($P < 0.05$), whereas SC kits showed intermediate values between Groups C and R for all morphometric parameters studied except fetal weight. Food restriction significantly increased the rate of newborn IUGR ($P < 0.05$; Table 5 and Fig. S3), whilst values obtained from the group with SC administration were intermediate between Groups C and R.

Discussion

The results of the present study in a rabbit model support the usefulness of treatment with sildenafil citrate to alleviate states of placental dysfunction and improve body size in fetuses affected by IUGR.

In the present trial, sildenafil citrate therapy favoured placental growth and vascularisation, lowered IUGR incidence and resulted in offspring with increased birth size (in terms of higher values of crown–rump length and biparietal and thoracic diameters). Our results support previous data showing that maternal undernutrition during pregnancy or defects in placental development have negative effects on fetal homeostasis and give way to the appearance of IUGR (Lesage *et al.* 2001; Pardi *et al.* 2002; Matsuoka *et al.* 2006). Herein we found that a 50% reduction in maternal food intake in a rabbit model impaired placental structural phenotype (reduced length of decidua and labyrinth compartments) and led to placental pathology (such as atrophy or fibrosis). In contrast, placentas from pregnancies treated with sildenafil citrate showed significant changes when compared with those in the restricted group at the labyrinth zone (higher values of labyrinth ratio and absence of fibrosis process) and at the decidua compartment (increase in thickness and significant hyperplasia and hypertrophy of arterial sinuses). We can hypothesise that these changes are related to two main factors. First, it is known that sildenafil citrate acts as a potent angiogenesis stimulator (Pyriochou *et al.* 2007), increasing the growth of new vessels at the labyrinth (which is supported by the histological assessment, showing a higher number of small dilated capillaries when compared with untreated placentas). Concomitantly, a recent study from Luna *et al.* (2015) also found slight vasodilatation at the labyrinth zone in placentas of mice treated with this therapy. Second, the myometrium and the decidua vessels express high levels of PDE-5 (Buhimschi *et al.* 2004; Coppage *et al.* 2005) and, in the case of the rabbit, the placenta

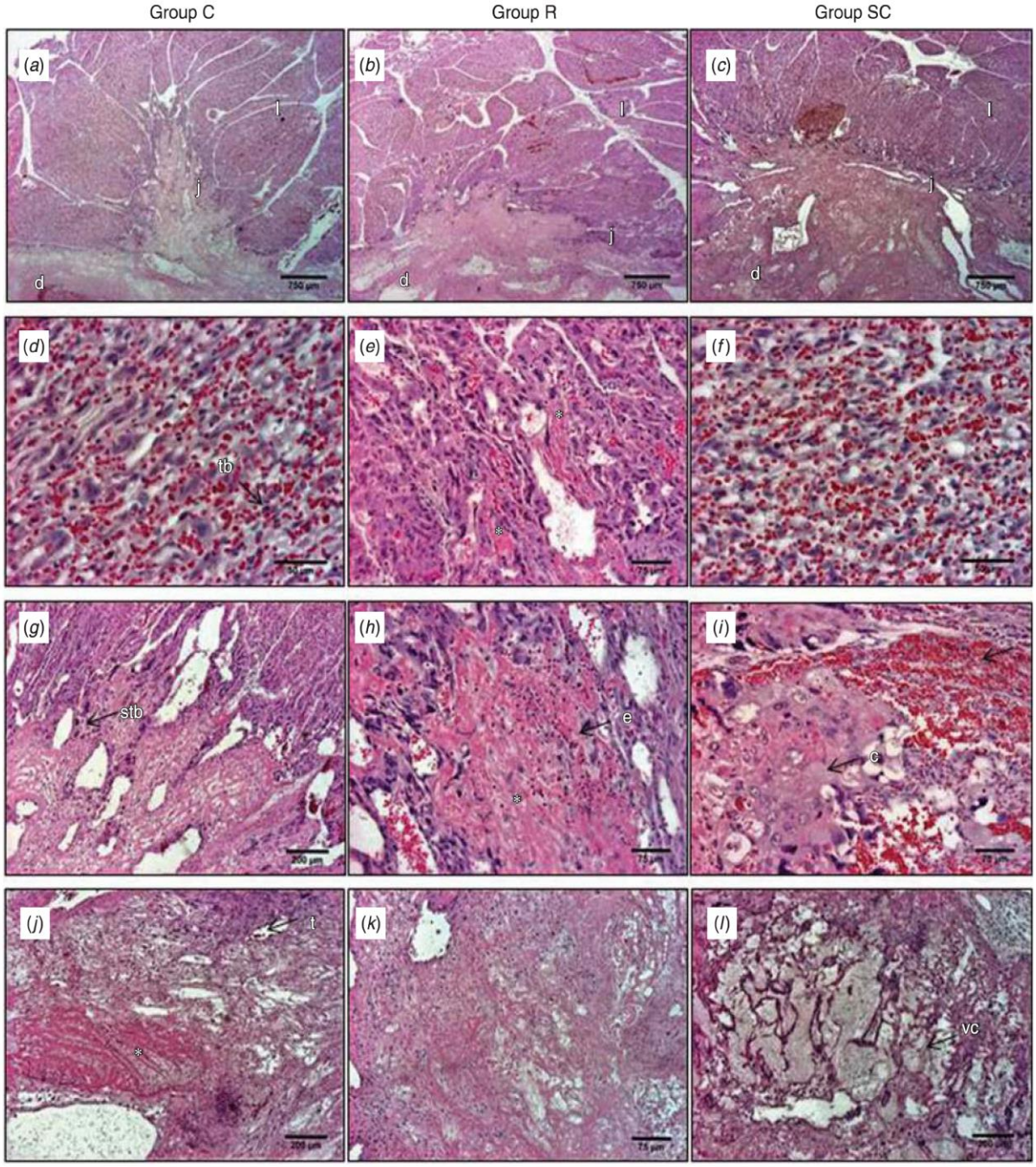

Fig. 1. Histological images of rabbit placenta at Day 28 of pregnancy in dams fed *ad libitum* (Group C), restricted diet (Group R) and restricted diet treated with sildenafil citrate (Group SC). (*a*, *b*, *c*) The three parts of the rabbit's placenta (l, labyrinth; j, junctional zone and d, decidua) in each of the three experimental groups. (*d*) Group C, normal trophoblast (tb) proliferation at the labyrinth zone. (*e*) Group R, vascular channels collapsed with multifocal areas of fibrosis (*) and mineralisation at the labyrinth zone. (*f*) Group SC, normal trophoblast proliferation at the labyrinth zone in a similar pattern to Group C. (*g*) Group C, vascularisation at the junctional zone with normal trophoblast and syncytiotrophoblasts (stb). (*h*) Group R, focus of sclerosis (*) at the junctional zone with inflammatory infiltrations (e) and decreased number of trophoblasts. (*i*) Group SC, junctional zone with syncytiotrophoblasts, increased vasculature with congestion (c) and haemorrhagic foci. (*j*) Group C, normal limits of the decidua with thrombi (t) mineral and inflammation (*). (k) Group R, decidua with necrosis, fibrin and few vascular channels. (*l*) Group SC, decidua with numerous dilated vascular channels (vc).

Table 4. Feto–placental haemodynamics at Day 26 of pregnancy from dams fed *ad libitum* (C), restricted diet (R) or restricted diet and treated with sildenafil citrate (SC)

Statistical analyses were performed by one-way ANOVA and *t*-test mean comparison test. Data represented as mean ± s.e.m. [a,b]Different superscripts within a row indicate significant differences between groups; $P < 0.05$

Parameter	C ($n = 16$)	R ($n = 16$)	SC ($n = 16$)	$P >$ f
Umbilical cord arteries				
Resistance index	0.70 ± 0.02	0.77 ± 0.01	0.77 ± 0.05	0.066
Pulsatility index	1.20 ± 0.05	1.25 ± 0.04	1.28 ± 0.05	0.468
Systolic peak velocity	33.20 ± 4.05^{a}	42.60 ± 3.46^{b}	43.90 ± 3.35^{b}	0.012
End diastolic velocity	8.00 ± 0.86	10.30 ± 1.16	10.30 ± 1.15	0.336
Time-averaged mean velocity	20.60 ± 2.37	26.40 ± 2.25	27.10 ± 2.17	0.137
Middle cerebral artery				
Resistance index	0.60 ± 0.02^{a}	0.60 ± 0.02^{a}	0.70 ± 0.02^{b}	0.023
Pulsatility index	0.90 ± 0.05^{a}	0.90 ± 0.05^{a}	1.10 ± 0.05^{b}	0.013
Systolic peak velocity	17.10 ± 2.21^{a}	22.20 ± 1.94^{b}	26.30 ± 2.02^{b}	0.015
End diastolic velocity	6.50 ± 0.63	8.30 ± 0.78	7.70 ± 0.46	0.165
Time-averaged mean velocity	11.80 ± 1.40^{a}	15.30 ± 1.28^{b}	17.00 ± 1.13^{b}	0.026

Table 5. Morphometric measurements of newborns from dams fed *ad libitum* (C), restricted diet (R) or restricted diet and treated with sildenafil citrate (SC)

Statistical analyses were performed by one-way ANOVA and *t*-test mean comparison test. Data represented as mean ± s.e.m. IUGR rate defined as those fetuses under 10th percentile of *ad libitum* control weights (37.9 g) estimated by a χ^2 test. [a,b,c]Different superscripts within a row indicate significant differences between groups; $P < 0.05$

Parameter	C ($n = 85$)	R ($n = 75$)	SC ($n = 76$)	$P >$ f
Bodyweight (g)	55.23 ± 1.11^{a}	49.46 ± 1.18^{b}	47.80 ± 1.19^{b}	0.001
Biparietal diameter (cm)	2.20 ± 0.01^{a}	2.08 ± 0.01^{b}	2.15 ± 0.01^{c}	0.001
Crown–rump length (cm)	11.00 ± 0.09^{a}	10.20 ± 0.09^{b}	10.50 ± 0.10^{c}	0.001
Thoracic diameter (cm)	2.40 ± 0.02^{a}	2.17 ± 0.02^{b}	2.28 ± 0.02^{c}	0.001
IUGR rate (%)	9.41^{a}	38.66^{b}	25.00^{c}	0.001

has a high expression level of NOS, especially NOS3 (Khan *et al.* 2012), which may facilitate sildenafil citrate function. Both processes would be expected to stimulate neoangiogenesis and may also improve maternal blood flow to the placenta, thereby facilitating nutrient and oxygen delivery to the fetus.

Consequently, fetal growth was improved in terms of crown–rump length and biparietal and thoracic diameters, at Day 28 and at birth. These findings support previous results from Sánchez-Aparicio *et al.* (2008) and Stanley *et al.* (2012). However, this larger body size was not concomitant with increases in fetal weight, supporting data from previous studies in rodent models (Ramesar *et al.* 2010; George *et al.* 2013; Motta *et al.* 2015). Notwithstanding, studies in sheep with 50% food restriction (similar to our restriction) have shown that sildenafil citrate treatment increased fetal weight by 14% (Satterfield *et al.* 2010). Such disagreement may be related to differences in nutrient partitioning between monotocous and polytocous species (Fowden and Moore 2012), the capacity of the placenta to adapt its phenotype or function to undernutrition and the length of sildenafil citrate therapy (a total of 87 days, from Day 28 to Day 115 of pregnancy, Satterfield *et al.* 2010).

A remarkable finding of this novel study using a rabbit model comes from the results obtained by assessing the relative growth of fetal organs with respect to fetal weight, which may set the basis for future studies on the use of sildenafil citrate therapies and its impact on fetal organs. Fetuses treated with sildenafil citrate developed a proportionally larger liver with respect to the other two groups. This observation is in line with results obtained in rats (Pellicer *et al.* 2011). It is well known that from early development the liver is vital for health and body physiology. It participates in fat deposition (Godfrey *et al.* 2012), regulates growth and metabolism by modulating hormones and growth factors (Hellerstein and Munro 1994; Tchirikov *et al.* 2002) and is responsible for gluconeogenesis (Burns *et al.* 1997), the latter of which could explain the high glucose level found in the SC group. But, even more importantly, the liver can also modulate blood distribution as it is the first organ to receive blood from the placenta (Tchirikov *et al.* 2002) and, due to the presence of the ductus venosus, may distribute blood towards essential organs at the expense of less-essential organs (Cohn *et al.* 1974; Jensen *et al.* 1991). As a result of alterations in the liver, blood distribution may have changed, favouring developmental adaptation in the fetus by selectively increasing blood flow to vital organs like the brain ('brain-sparing effect').

The data obtained in the present study support the idea that undernutrition early in pregnancy may affect vital organs leading to disproportionate growth of the fetus (Bauer *et al.* 2003; Desai *et al.* 2007) and that the fetus can counteract this by an innate mechanism of fetal cardiac output distribution (Giussani 2011). In the present study, restricted fetuses showed higher head and brain mass relative to bodyweight, which suggests asymmetric growth retardation of the fetus, supporting the idea of the 'thrifty phenotype' (Wells 2011). Also, these data support previous studies in which the comparison of the brain weight to liver weight ratio was associated with undernutrition and dysmaturity (Anderson 1972; Camm *et al.* 2010). However,

the results obtained by dividing these weights suggest that therapy with sildenafil citrate could ameliorate this ratio. Another finding in this study is that, although this brain-sparing effect has been proposed to be stimulated by the hypoglycaemic status in the restricted fetus (Giussani 2011), data obtained in this rabbit model suggest that there may be factors in addition to fetal glycaemic status that contribute.

Assessment of blood flow by Doppler ultrasonography at Day 26 of pregnancy showed that food restriction induced changes in the haemodynamic patterns of the fetus. Those IUGR fetuses exhibited a trend to increase the umbilical artery resistance index and demonstrated a significant increase in the systolic peak velocity, suggesting a deterioration of placental function (Carr *et al.* 2012). These blood-flow changes could not be rescued by sildenafil citrate, which is contrary to data from previous studies (Dastjerdi *et al.* 2012; Lin *et al.* 2012; Stanley *et al.* 2012; Trapani *et al.* 2015). As a consequence of the placental dysfunction and reduction in oxygen levels, the middle cerebral artery in both restricted and sildenafil-treated fetuses exhibited changes in the systolic mean velocity and therefore increased mean velocity values, which agrees with previous data on the middle cerebral artery of fetuses affected by IUGR (Hanif *et al.* 2007; Mari *et al.* 2007).

Nevertheless, fetuses undergoing sildenafil citrate therapy showed elevated values of pulsatility and resistance indexes, as Dastjerdi *et al.* (2012) found in pregnant women, which may suggest a certain grade of vasoconstriction (low indices reflects redistribution of cardiac output to the brain; Mari *et al.* 2007). It is known that cerebral neurons and vessels have high concentrations of PDE-5 (Kotera *et al.* 2000; Lin *et al.* 2006) and that sildenafil citrate can cross the placenta (Pellicer *et al.* 2011). Moreover, this therapy can increase brain cGMP levels (Zhang *et al.* 2002) and cerebral blood flow (Li *et al.* 2007). Taken together, these data suggest that fetuses from sildenafil-treated mothers activate a protective mechanism in the middle cerebral artery to counteract an excess in blood-flow supply that could produce cerebral oedema and consequent adverse neurological outcomes. However, these data should be interpreted with caution, as the Doppler assessment was only performed once during the pregnancy, and thus other possible changes in gestation could not be determined in response to sildenafil citrate in this study. Further studies are needed to determine the possible risks of blood overflow in the fetal brain and also to identify the mechanism by which the fetus is able to adapt its cerebral arterial vascular tone; this process possibly depends on the enhancement of nitric oxide abundance with sildenafil citrate administration. Furthermore, elucidating whether these haemodynamic and morphologic adaptations of the fetus can have consequences in adult life should be the focus of future investigations.

In summary, the results of the present study suggest that, in rabbits, a 50% restriction of maternal food intake is a valid model for inducing IUGR and placental insufficiency. Such effects can be partially counteracted by the administration of sildenafil citrate, since it improves reductions in perinatal body size and modifies placental growth and vascularisation in the labyrinth and decidua. Therefore, size of the newborns can be partially improved. Thus, our study sets the basis of further studies investigating the use of PDE-5 inhibitors to study organ development and the programming of offspring growth and postnatal homeostasis (in particular brain and liver function).

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References

Anderson, J. M. (1972). Increased brain weight–liver weight ratio as a necropsy sign of intrauterine undernutrition. *J. Clin. Pathol.* **25**, 867–871. doi:10.1136/JCP.25.10.867

Bauer, R., Walter, B., Brust, P., Fuchtner, F., and Zwiener, U. (2003). Impact of asymmetric intrauterine growth restriction on organ function in newborn piglets. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **110**(Suppl 1), S40–S49. doi:10.1016/S0301-2115(03)00171-4

Beaudoin, S., Barbet, P., and Bargy, F. (2003). Developmental stages in the rabbit embryo: guidelines to choose an appropriate experimental model. *Fetal Diagn. Ther.* **18**, 422–427. doi:10.1159/000073136

Buhimschi, C. S., Garfield, R. E., Weiner, C. P., and Buhimschi, I. A. (2004). The presence and function of phosphodiesterase type 5 in the rat myometrium. *Am. J. Obstet. Gynecol.* **190**, 268–274. doi:10.1016/J.AJOG.2003.07.006

Burns, S. P., Desai, M., Cohen, R. D., Hales, C. N., Iles, R. A., Germain, J. P., Going, T. C., and Bailey, R. A. (1997). Gluconeogenesis, glucose handling and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation. *J. Clin. Invest.* **100**, 1768–1774. doi:10.1172/JCI119703

Camm, E. J., Hansell, J. A., Kane, A. D., Herrera, E. A., Lewis, C., Wong, S., Morrell, N. W., and Giussani, D. A. (2010). Partial contributions of developmental hypoxia and undernutrition to prenatal alterations in somatic growth and cardiovascular structure and function. *Am. J. Obstet. Gynecol.* **203**, 495.e24–495.e34. doi:10.1016/J.AJOG.2010.06.046

Carr, D. J., Aitken, R. P., Milne, J. S., David, A. L., and Wallace, J. M. (2012). Feto–placental biometry and umbilical artery Doppler velocimetry in the overnourished adolescent model of fetal growth restriction. *Am. J. Obstet. Gynecol.* **207**, 141.e6–141.e15. doi:10.1016/J.AJOG.2012.05.008

Cauli, O., Herraiz, S., Pellicer, B., Pellicer, A., and Felipo, V. (2010). Treatment with sildenafil prevents impairment of learning in rats born to pre-eclamptic mothers. *Neuroscience* **171**, 506–512. doi:10.1016/J.NEUROSCIENCE.2010.08.065

Chuang, A. T., Strauss, J. D., Murphy, R. A., and Steers, W. D. (1998). Sildenafil, a type-5 cGMP phosphodiesterase inhibitor, specifically amplifies endogenous cGMP-dependent relaxation in rabbit corpus cavernosum smooth muscle *in vitro*. *J. Urol.* **160**, 257–261. doi:10.1016/S0022-5347(01)63100-8

Cohn, H. E., Sacks, E. J., Heymann, M. A., and Rudolph, A. M. (1974). Cardiovascular responses to hypoxemia and acidemia in fetal lambs. *Am. J. Obstet. Gynecol.* **120**, 817–824. doi:10.1016/0002-9378(74)90587-0

Coppage, K. H., Sun, X., Baker, R. S., and Clark, K. E. (2005). Expression of phosphodiesterase 5 in maternal and fetal sheep. *Am. J. Obstet. Gynecol.* **193**, 1005–1010. doi:10.1016/J.AJOG.2005.05.054

Dastjerdi, M. V., Hosseini, S., and Bayani, L. (2012). Sildenafil citrate and utero–placental perfusion in fetal growth restriction. *J. Res. Med. Sci.* **17**, 632–636.

Derrick, M., Drobyshevsky, A., Ji, X., Chen, L., Yang, Y., Ji, H., Silverman, R. B., and Tan, S. (2009). Hypoxia–ischemia causes persistent movement deficits in a perinatal rabbit model of cerebral palsy: assessed by a new swim test. *Int. J. Dev. Neurosci.* **27**, 549–557. doi:10.1016/J.IJDEVNEU.2009.06.008

Desai, M., Gayle, D., Babu, J., and Ross, M. G. (2007). The timing of nutrient restriction during rat pregnancy/lactation alters metabolic syndrome phenotype. *Am. J. Obstet. Gynecol.* **196**, 555.e1–555.e7. doi:10.1016/J.AJOG.2006.11.036

Dilworth, M. R., Andersson, I., Renshall, L. J., Cowley, E., Baker, P., Greenwood, S., Sibley, C. P., and Wareing, M. (2013). Sildenafil citrate increases fetal weight in a mouse model of fetal growth restriction with a normal vascular phenotype. *PLoS One* **8**, e77748. doi:10.1371/JOURNAL.PONE.0077748

Eixarch, E., Figueras, F., Hernandez-Andrade, E., Crispi, F., Nadal, A., Torre, I., Oliveira, S., and Gratacos, E. (2009). An experimental model of fetal growth restriction based on selective ligature of utero–placental vessels in the pregnant rabbit. *Fetal Diagn. Ther.* **26**, 203–211. doi:10.1159/000264063

Fischer, B., Chavatte-Palmer, P., Viebahn, C., Navarrete Santos, A., and Duranthon, V. (2012). Rabbit as a reproductive model for human health. *Reproduction* **144**, 1–10. doi:10.1530/REP-12-0091

Fowden, A. L., and Moore, T. (2012). Maternal–fetal resource allocation: co-operation and conflict. *Placenta* **33**(Suppl 2), e11–e15. doi:10.1016/J.PLACENTA.2012.05.002

Ganzevoort, W., Alfirevic, Z., von Dadelszen, P., Kenny, L., Papageorghiou, A., van Wassenaer-Leemhuis, A., Gluud, C., Mol, B. W., and Baker, P. N. (2014). STRIDER: Sildenafil therapy in dismal prognosis early-onset intrauterine growth restriction – a protocol for a systematic review with individual participant data and aggregate data meta-analysis and trial sequential analysis. *Syst. Rev.* **3**, 23. doi:10.1186/2046-4053-3-23

George, E. M., Palei, A. C., Dent, E. A., and Granger, J. P. (2013). Sildenafil attenuates placental ischemia-induced hypertension. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **305**, R397–R403. doi:10.1152/AJPREGU.00216.2013

Ghidini, A. (1996). Idiopathic fetal growth restriction: a pathophysiologic approach. *Obstet. Gynecol. Surv.* **51**, 376–382. doi:10.1097/00006254-199606000-00023

Giussani, D. A. (2011). The vulnerable developing brain. *Proc. Natl. Acad. Sci. USA* **108**, 2641–2642. doi:10.1073/PNAS.1019726108

Godfrey, K. M., Haugen, G., Kiserud, T., Inskip, H. M., Cooper, C., Harvey, N. C., Crozier, S. R., Robinson, S. M., Davies, L., and Hanson, M. A. (2012). Fetal liver blood-flow distribution: role in human developmental strategy to prioritise fat deposition versus brain development. *PLoS One* **7**, e41759. doi:10.1371/JOURNAL.PONE.0041759

Hanif, F., Drennan, K., and Mari, G. (2007). Variables that affect the middle cerebral artery peak systolic velocity in fetuses with anaemia and intrauterine growth restriction. *Am. J. Perinatol.* **24**, 501–505. doi:10.1055/S-2007-986683

Hellerstein, M. K., and Munro, H. N. (1994). Interaction of liver, muscle and adipose tissue in the regulation of metabolism in response to nutritional and other factors. In 'The Liver: Biology and Pathobiology'. (Ed(s) I. M. Arias, J. L. Boyer, N. Fausto.) pp. 1169–1191. (Raven Press: New York.)

Jensen, A., Roman, C., and Rudolph, A. M. (1991). Effects of reducing uterine blood flow on fetal blood-flow distribution and oxygen delivery. *J. Dev. Physiol.* **15**, 309–323.

Khan, H., Kusakabe, K. T., Wakitani, S., Hiyama, M., Takeshita, A., and Kiso, Y. (2012). Expression and localisation of NO synthase isoenzymes (iNOS and eNOS) in development of the rabbit placenta. *J. Reprod. Dev.* **58**, 231–236. doi:10.1262/JRD.11-128T

Kobayashi, T., Ito, T., and Shiomi, M. (2011). Roles of the WHHL rabbit in translational research on hypercholesterolemia and cardiovascular diseases. *J. Biomed. Biotechnol.* **2011**, 406473. doi:10.1155/2011/406473

Kotera, J., Fujishige, K., and Omori, K. (2000). Immunohistochemical localisation of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in rat tissues. *J. Histochem. Cytochem.* **48**, 685–693. doi:10.1177/002215540004800512

Lacassie, H. J., Germain, A. M., Valdes, G., Fernandez, M. S., Allamand, F., and Lopez, H. (2004). Management of Eisenmenger syndrome in pregnancy with sildenafil and L-arginine. *Obstet. Gynecol.* **103**, 1118–1120. doi:10.1097/01.AOG.0000125148.82698.65

Lecarpentier, E., Morel, O., Tarrade, A., Dahirel, M., Bonneau, M., Gayat, E., Evain-Brion, D., Chavatte-Palmer, P., and Tsatsaris, V. (2012). Quantification of utero–placental vascularisation in a rabbit model of IUGR with three-dimensional power Doppler angiography. *Placenta* **33**, 769–775. doi:10.1016/J.PLACENTA.2012.06.013

Lesage, J., Blondeau, B., Grino, M., Breant, B., and Dupouy, J. P. (2001). Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo–pituitary–adrenal axis in the newborn rat. *Endocrinology* **142**, 1692–1702.

Li, L., Jiang, Q., Zhang, L., Ding, G., Gang Zhang, Z., Li, Q., Ewing, J. R., Lu, M., Panda, S., Ledbetter, K. A., Whitton, P. A., and Chopp, M. (2007). Angiogenesis and improved cerebral blood flow in the ischemic boundary area detected by MRI after administration of sildenafil to rats with embolic stroke. *Brain Res.* **1132**, 185–192. doi:10.1016/J.BRAINRES.2006.10.098

Lin, C. S., Lin, G., Xin, Z. C., and Lue, T. F. (2006). Expression, distribution and regulation of phosphodiesterase 5. *Curr. Pharm. Des.* **12**, 3439–3457. doi:10.2174/138161206778343064

Lin, T. H., Su, Y. N., Shih, J. C., Hsu, H. C., and Lee, C. N. (2012). Resolution of high uterine artery pulsatility index and notching following sildenafil citrate treatment in a growth-restricted pregnancy. *Ultrasound Obstet. Gynecol.* **40**, 609–610. doi:10.1002/UOG.11142

López-Tello, J., Barbero, A., González-Bulnes, A., Astiz, S., Rodríguez, M., Formoso-Rafferty, N., Arias-Álvarez, M., and Rebollar, P. G. (2015). Characterisation of early changes in feto–placental haemodynamics in a diet-induced rabbit model of IUGR. *J. Dev. Orig. Health Dis.* **6**, 454–461. doi:10.1017/S2040174415001385

Luna, R. L., Nunes, A. K., Oliveira, A. G., Araujo, S. M., Lemos, A. J., Rocha, S. W., Croy, B. A., and Peixoto, C. A. (2015). Sildenafil (Viagra) blocks inflammatory injury in LPS-induced mouse abortion: a potential prophylactic treatment against acute pregnancy loss? *Placenta* **36**, 1122–1129. doi:10.1016/J.PLACENTA.2015.07.133

Malassiné, A., Frendo, J. L., and Evain-Brion, D. (2003). A comparison of placental development and endocrine functions between the human and mouse model. *Hum. Reprod. Update* **9**, 531–539. doi:10.1093/HUMUPD/DMG043

Mari, G., Hanif, F., Kruger, M., Cosmi, E., Santolaya-Forgas, J., and Treadwell, M. C. (2007). Middle cerebral artery peak systolic velocity: a new Doppler parameter in the assessment of growth-restricted fetuses. *Ultrasound Obstet. Gynecol.* **29**, 310–316. doi:10.1002/UOG.3953

Maršál, K. (2002). Intrauterine growth restriction. *Curr. Opin. Obstet. Gynecol.* **14**, 127–135. doi:10.1097/00001703-200204000-00005

Matsuoka, T., Mizoguchi, Y., Serizawa, K., Ishikura, T., Mizuguchi, H., and Asano, Y. (2006). Effects of stage and degree of restricted feeding on pregnancy outcome in rabbits. *J. Toxicol. Sci.* **31**, 169–175. doi:10.2131/JTS.31.169

Motta, C., Grosso, C., Zanuzzi, C., Molinero, D., Picco, N., Bellingeri, R., Alustiza, F., Barbeito, C., Vivas, A., and Romanini, M. C. (2015). Effect of Sildenafil on pre-eclampsia-like mouse model induced by L-name. *Reprod. Domest. Anim.* **50**, 611–616. doi:10.1111/RDA.12536

Nardozza, L. M., Araujo Junior, E., Barbosa, M. M., Caetano, A. C., Lee, D. J., and Moron, A. F. (2012). Fetal growth restriction: current knowledge to the general Obs/Gyn. *Arch. Gynecol. Obstet.* **286**, 1–13. doi:10.1007/S00404-012-2330-6

Panda, S., Das, A., and Nowroz, H. M. (2014). Sildenafil citrate in fetal growth restriction. *J. Reprod. Infertil.* **15**, 168–169.

Pardi, G., Marconi, A. M., and Cetin, I. (2002). Placental–fetal interrelationship in IUGR fetuses – a review. *Placenta* **23**, S136–S141. doi:10.1053/PLAC.2002.0802

Park, J. Y., Son, H., Kim, S. W., and Paick, J. S. (2004). Potentiation of apomorphine effect on sildenafil-induced penile erection in conscious rabbits. *Asian J. Androl.* **6**, 205–209.

Pellicer, B., Herraiz, S., Cauli, O., Rodrigo, R., Asensi, M., Cortijo, J., Serra, V., Morcillo, E., Felipo, V., Simón, C., and Pellicer, A. (2011). Haemodynamic effects of long-term administration of sildenafil in normotensive pregnant and non-pregnant rats. *BJOG* **118**, 615–623. doi:10.1111/J.1471-0528.2010.02839.X

Polisca, A., Scotti, L., Orlandi, R., Brecchia, G., and Boiti, C. (2010). Doppler evaluation of maternal and fetal vessels during normal gestation in rabbits. *Theriogenology* **73**, 358–366. doi:10.1016/J.THERIOGENOLOGY.2009.09.019

Purcell, T. L., Given, R., Chwalisz, K., and Garfield, R. E. (1999). Nitric oxide synthase distribution during implantation in the mouse. *Mol. Hum. Reprod.* **5**, 467–475. doi:10.1093/MOLEHR/5.5.467

Püschel, B., Daniel, N., Bitzer, E., Blum, M., Renard, J. P., and Viebahn, C. (2010). The rabbit (*Oryctolagus cuniculus*): a model for mammalian reproduction and early embryology. *Cold Spring Harb. Protoc.* doi:10.1101/PDB.EMO139

Pyriochou, A., Zhou, Z., Koika, V., Petrou, C., Cordopatis, P., Sessa, W. C., and Papapetropoulos, A. (2007). The phosphodiesterase 5 inhibitor sildenafil stimulates angiogenesis through a protein kinase G/MAPK pathway. *J. Cell. Physiol.* **211**, 197–204. doi:10.1002/JCP.20929

Ramesar, S. V., Mackraj, I., Gathiram, P., and Moodley, J. (2010). Sildenafil citrate improves fetal outcomes in pregnant, L-NAME-treated, Sprague–Dawley rats. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **149**, 22–26. doi:10.1016/J.EJOGRB.2009.11.005

Rebollar, P. G., Dal Bosco, A., Millán, P., Cardinali, R., Brecchia, G., Sylla, L., Lorenzo, P. L., and Castellini, C. (2012). Ovulating induction methods in rabbit does: the pituitary and ovarian responses. *Theriogenology* **77**, 292–298. doi:10.1016/J.THERIOGENOLOGY.2011.07.041

Reynolds, S. R. (1946). The relation of hydrostatic conditions in the uterus to the size and shape of the conceptus during pregnancy; a concept of uterine accommodation. *Anat. Rec.* **95**, 283–296. doi:10.1002/AR.1090950303

Ross, M. G., and Desai, M. (2013). Developmental programming of offspring obesity, adipogenesis and appetite. *Clin. Obstet. Gynecol.* **56**, 529–536. doi:10.1097/GRF.0B013E318299C39D

Samuel, C. A., Jack, P. M., and Nathanielsz, P. W. (1975). Ultrastructural studies of the rabbit placenta in the last third of gestation. *J. Reprod. Fertil.* **45**, 9–14. doi:10.1530/JRF.0.0450009

Sánchez-Aparicio, P., Mota-Rojas, D., Nava-Ocampo, A. A., Trujillo-Ortega, M. E., Alfaro-Rodríguez, A., Arch, E., and Alonso-Spilsbury, M. (2008). Effects of sildenafil on the fetal growth of guinea pigs and their ability to survive induced intrapartum asphyxia. *Am. J. Obstet. Gynecol.* **198**, 127.e1–127.e6. doi:10.1016/J.AJOG.2007.06.068

Sankaran, S., and Kyle, P. M. (2009). Aetiology and pathogenesis of IUGR. *Best Pract. Res. Clin. Obstet. Gynaecol.* **23**, 765–777. doi:10.1016/J.BPOBGYN.2009.05.003

Satterfield, M. C., Bazer, F. W., Spencer, T. E., and Wu, G. (2010). Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. *J. Nutr.* **140**, 251–258. doi:10.3945/JN.109.114678

Schroder, H. J. (2003). Models of fetal growth restriction. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **110**(Suppl 1), S29–S39. doi:10.1016/S0301-2115(03)00170-2

Serrano, N. C., Casas, J. P., Diaz, L. A., Paez, C., Mesa, C. M., Cifuentes, R., Monterrosa, A., Bautista, A., Hawe, E., Hingorani, A. D., Vallance, P., and Lopez-Jaramillo, P. (2004). Endothelial NO synthase genotype and risk of pre-eclampsia: a multicentre case-control study. *Hypertension* **44**, 702–707. doi:10.1161/01.HYP.0000143483.66701.EC

Stanley, J. L., Andersson, I. J., Poudel, R., Rueda-Clausen, C. F., Sibley, C. P., Davidge, S. T., and Baker, P. N. (2012). Sildenafil citrate rescues fetal growth in the catechol-o-methyl transferase knockout mouse model. *Hypertension* **59**, 1021–1028. doi:10.1161/HYPERTENSIONAHA.111.186270

Sun, X., Wang, K., Wang, W., and Li, B. (2014). Clinical study on sildenafil in treatment of pregnant women with pulmonary arterial hypertension. *Zhonghua Fu Chan Ke Za Zhi* **49**, 414–418.

Tchirikov, M., Kertschanska, S., Sturenberg, H. J., and Schroder, H. J. (2002). Liver blood perfusion as a possible instrument for fetal growth regulation. *Placenta* **23**(Suppl A), S153–S158. doi:10.1053/PLAC.2002.0810

Trapani, A. J., Goncalves, L. F., Trapani, T. F., Franco, M. J., Galluzzo, R. N., and Pires, M. M. (2015). Comparison between transdermal nitroglycerin and sildenafil citrate in intrauterine growth restriction: effect on uterine, umbilical and fetal middle cerebral artery pulsatility index. *Ultrasound Obstet. Gynecol.* doi:10.1002/UOG.15673

Wells, J. C. (2011). The thrifty phenotype: an adaptation in growth or metabolism? *Am. J. Hum. Biol.* **23**, 65–75. doi:10.1002/AJHB.21100

Zhang, R., Wang, Y., Zhang, L., Zhang, Z., Tsang, W., Lu, M., and Chopp, M. (2002). Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke* **33**, 2675–2680. doi:10.1161/01.STR.0000034399.95249.59

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Supplementary Material: Reproduction, Fertility and Development

Supplementary Material

The effects of sildenafil citrate on feto–placental development and haemodynamics in a rabbit model of intrauterine growth restriction

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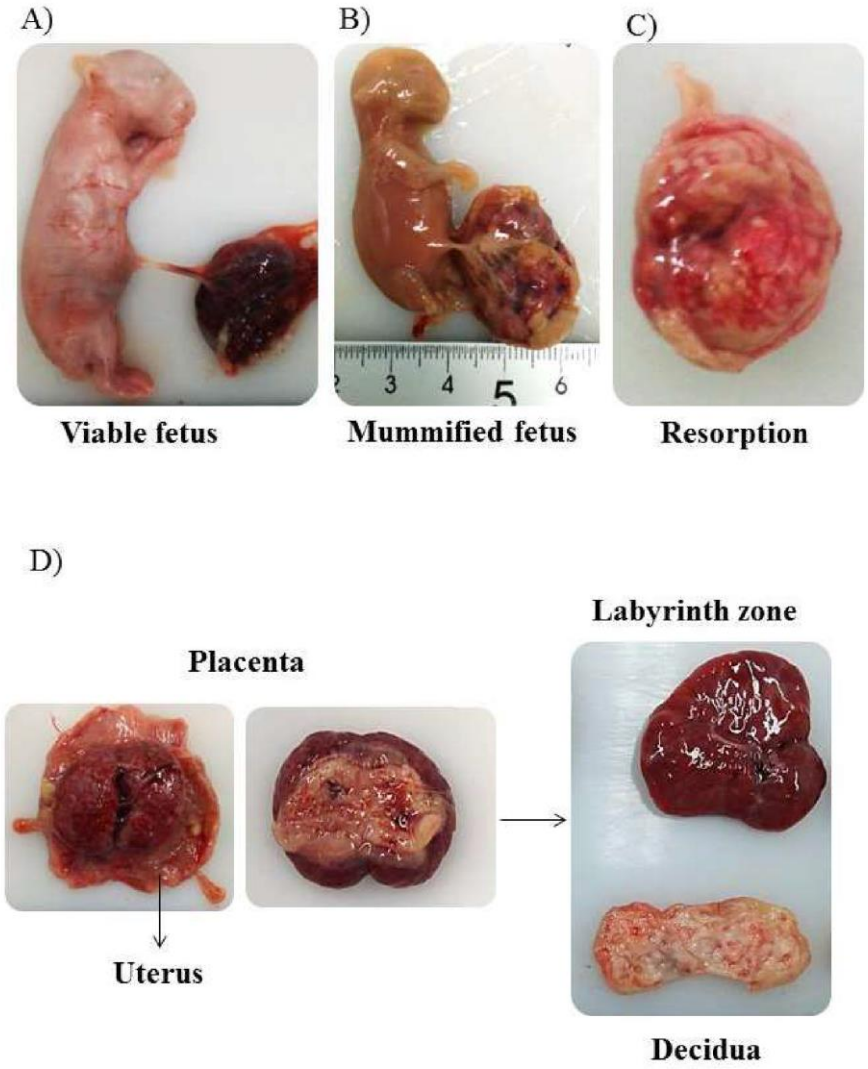

Fig. S1. Macroscopic image of rabbit conceptus at Day 28 of pregnancy. (A) Viable fetus; (B) mummified fetus; (C) resorption; and (D) rabbit placenta with their corresponding separating zones, labyrinth and decidua.

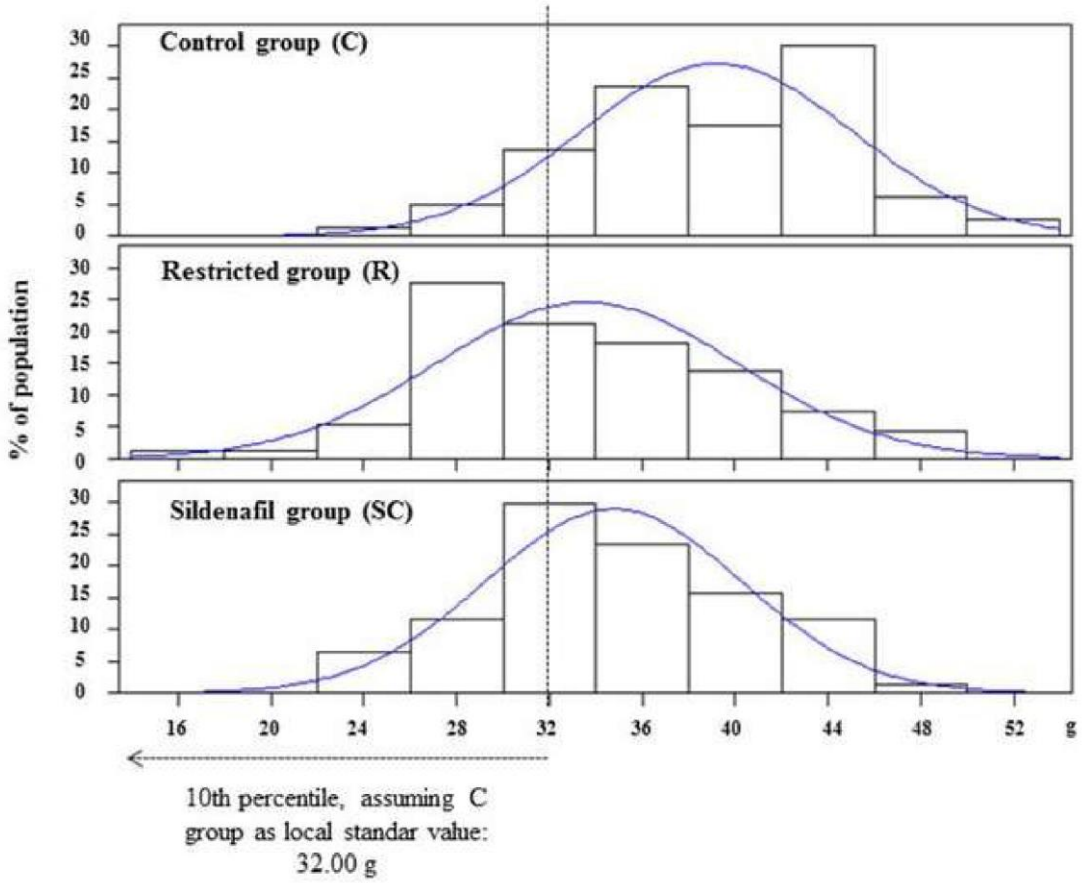

Fig. S2. Distribution curve of viable fetuses at Day 28 of pregnancy from dams fed ad libitum (C = 7), restricted (R = 8) or restricted and treated with sildenafil citrate (SC = 7). Vertical dashed line denotes the 10th percentile of control weights (32 g). Number of fetuses per treatment: C group, n = 81; R group, n = 94; SC group, n = 77.

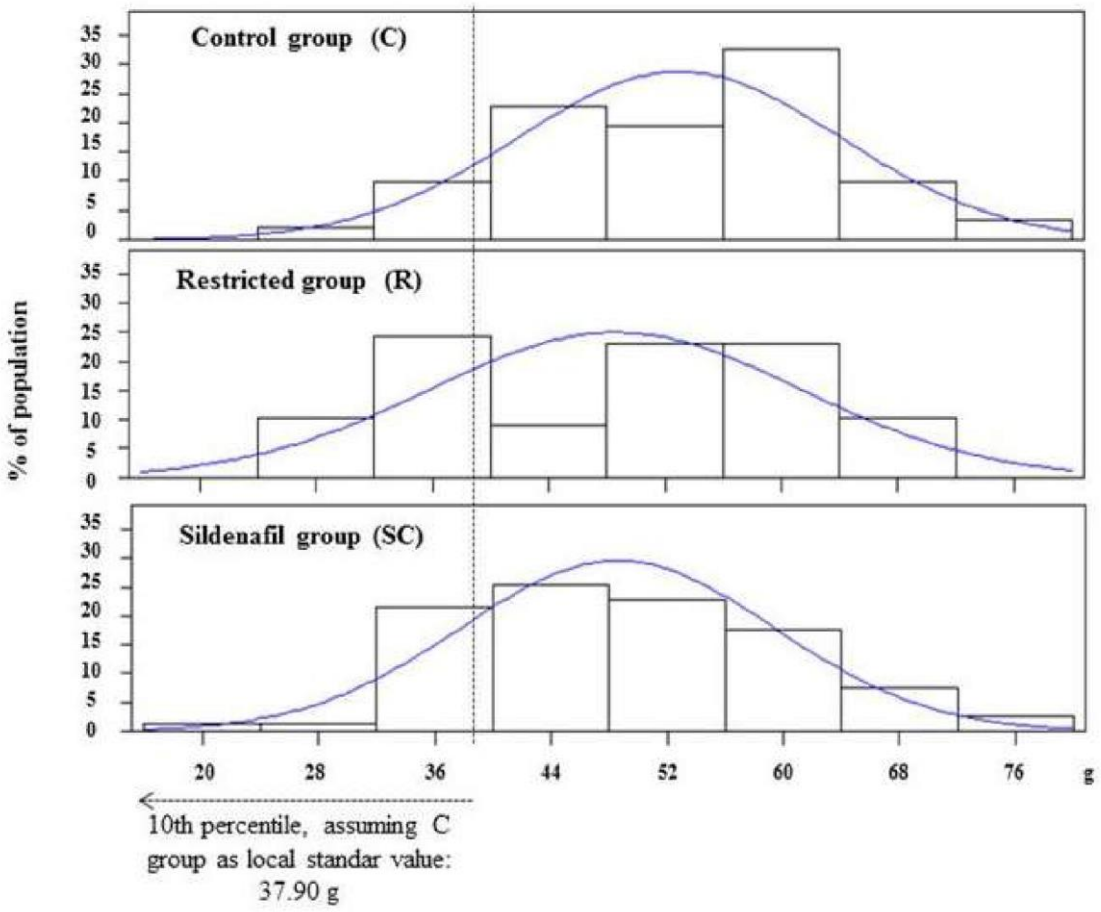

Fig. S3. Distribution curve of newborns at birth from dams fed ad libitum (C = 8), restricted (R = 7) or restricted and treated with sildenafil citrate (SC = 8). Vertical dashed line denotes the 10th percentile of ad libitum control weights (37.90 g). Number of newborns per treatment: C group, n = 85; R group, n = 75; SC group, n = 76.

Competition for materno-fetal resource partitioning in a rabbit model of undernourished pregnancy

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COMPETITION FOR MATERNO-FETAL RESOURCE PARTITIONING IN A RABBIT MODEL OF UNDERNOURISHED PREGNANCY

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Short Title: Food restriction during rabbit pregnancy

Keywords: Malnutrition, Metabolism, Fetus, Placenta, Apoptosis

ABSTRACT

The major goal of animal production is to obtain abundant and healthy meat for consumers. Maternal food restriction (MFR) is often applied in farms to reduce production costs. However, the suitability of MFR in livestock animals is questionable, as this management may compromise maternal fitness due to a severe negative energetic balance and can induce Intrauterine Growth Restriction (IUGR) and prenatal programming in the offspring. Here, we sought to determine, using pregnant rabbits, the consequences of MFR on maternal endocrine and metabolic status and conceptus development. Pregnant dams were distributed into three groups: CONTROL (*ad libitum* feeding throughout the entire pregnancy; mean pregnancy length being around 31 days), UNDERFED (50% MFR during the entire pregnancy) and EARLY-UNDERFED (50% MFR only during the preimplantation period, Days 0-7). Maternal leptin concentrations and glycemic and lipid profiles were determined throughout pregnancy, whilst conceptus development was assessed *ex vivo* at Day 28. Placental parameters were determined by macroscopic and histological evaluations and apoptotic assessments (TUNEL and Caspase-3). The main results of the study showed that, despite MFR altered maternal plasma lipid concentration ($P<0.05$), there were no effects on maternal bodyweight, plasma leptin concentration or glycemic profile. Fetal crown-rump lengths were reduced in both undernourished groups ($P<0.001$), but a significant reduction in fetal weight was only observed in the UNDERFED group ($P<0.001$). Growth in both undernourished groups was asymmetrical, with reduced liver weight ($P<0.001$) and significantly increased brain: fetal weight-ratio ($P<0.001$) and brain: liver weight-ratio ($P<0.001$) when compared to the CONTROL group. A significant reduction in placental weight was only observed in the UNDERFED group ($P<0.001$), despite both undernourished groups showing higher apoptotic rates at decidua and labyrinth zone ($P<0.05$) than the CONTROL group. Thus, these groups evidenced signs of placental degeneration, necrosis and stromal collapse. In summary, MFR may encourage the mother to make strategic decisions to safeguard her metabolic status and fitness at the expense of growth reduction in the litter, resulting in enhanced apoptotic and pathological processes at placental level and IUGR.

INTRODUCTION

The major goal of animal production is to obtain abundant and healthy meat for consumers, which relies on adequate management of breeding animals and pregnancy periods [1]. In livestock animals, maternal nutrition has been largely recognized as a key factor for pregnancy success [1, 2]. Periods of maternal food restriction (MFR) during gestation can result in offspring suffering from Intrauterine Growth Restriction (IUGR), defined as the failure of a fetus to reach its genetic potential size [3]. The same situation has been found in humans, with MFR having a strong impact of IUGR occurrence and a higher incidence of non-communicable diseases, like obesity, cognitive dysfunctions or cardiometabolic disorders [4-6].

In livestock animals, the intensive productive rhythms and the different farm managements aiming to reduce productive costs have led to a higher occurrence of IUGR in these animals [1, 7]. Thereby, affecting the quality of their meat (muscle fibers and marbling), athletic performance or fleece production [2, 8] and lastly resulting in poorer incomes for the livestock producer and lower quality products for the consumers. Despite these inconveniences, MFR protocols applied in specific periods of the pregnancy, such as the preimplantation period, in which the embryo's requirements are low and the mother presents an anabolic status, could reduce productive costs and be an alternative strategy in farms [9, 10]. However, the pros and cons of such managements need to be further investigated, since inadequate nutrition from early gestation can influence placentation processes (specifically the allocation of trophectoderm and inner cell mass within the blastocyst [11]), which may impair placental development and function [12], compromising pregnancy outputs. In fact, experimental studies suggest that impaired placental structure or function (*e.g.* placental insufficiency) may contribute to IUGR in response to undernutrition [12, 13]. Most of these studies have been performed in rodents [14], whilst the use of large animals is scarce. However, large animals (sheep, pig or rabbit) offer a wider range of benefits for the purpose of this assessment, as the results obtained from these trials, especially those based on MFR protocols, can be useful not only for biomedicine but also to unravel the aforementioned pros and cons of the application of MFR regimens to livestock animals.

In the last years, the rabbit, considered as a livestock animal in the Mediterranean area (meat and fibre production [15, 16]), has emerged as a valuable model to investigate IUGR [17-31], as compared to a sheep or a pig, this animal does not need large animal facilities and gestational

length is shorter [term around Day (D) 31]. Thus, the rabbit develops a discoid hemochorial placentation and the fetuses have an accelerated perinatal brain growth, such characteristics are comparable with the human [32, 33]. Moreover, hemodynamic changes occurring during pregnancy are also similar to the human, with an important increase in maternal blood pressure throughout gestation [33, 34]. In rabbits, the effects of MFR during gestation [17, 30, 31, 35-41] vary depending on the period of the pregnancy exposed, the level of the restriction applied and the capacity of the mother to compensate, particularly during late pregnancy when fetal growth is maximal [42]. When it comes to the mother, MFR is usually linked to metabolic and hormonal changes [35-37] and low milk production [38]. Meanwhile, in the fetus, hemodynamic alterations and poor biometric outcomes can be observed [17, 30, 31, 39-41].

It has been previously shown using other animal species that MFR based on preconception or in *ad libitum* food intake can affect placental development [43], induce IUGR [44] and developmental programming of adult diseases in the offspring [45]. In previous work, using the rabbit as a model, we evaluated the effects of MFR based on *ad libitum* pre-pregnancy intake [30, 31]. However, in these studies, MFR was applied when the rabbit embryo was already implanted (from D9 to term). MFR resulted in fetal IUGR (measured on day 28 of pregnancy and at birth) despite no differences in placental weight or in perinatal mortality were observed. Here, we sought to determine the consequences of MFR applied only during the critical period of preimplantation (D0-D7) or throughout the gestation (D0 onwards) on: 1) maternal food intake, endocrine and metabolic status of the dams, 2) conceptus development at term and 3) placental homeostasis determined by histopathological study and apoptosis quantification.

MATERIALS AND METHODS

Animals and experimental design

Animals were housed in the facilities of the Polytechnic University of Madrid (UPM, Spain). All experiments were carried out in accordance with the National and Local rules of the Community of Madrid (Ref. PROEX 302/15), which meets the requirements of the Spanish policy for animal protection RD53/2013 and the European Union scientific procedures. Prior to the experimental phase (two weeks before mating), a total of 32 New Zealand x California rabbits (average 4.74 ± 0.12 Kg) were fed *ad libitum* with a diet containing 16% crude protein, 37% crude fiber, 3.7% fat and 2400 kcal/kg of crude energy (NANTA, Madrid, Spain). During this period the food

consumption of each dam was recorded daily. Dams were inseminated (D0 of pregnancy) with fresh diluted semen (commercial extender, MA 24, Ovejero, León, Spain). Each dose contained at least 25 million spermatozoa in 0.5 ml of diluent (Magapor S.L., Zaragoza, Spain). Ovulation was induced with gonadoreline at the time of mating (20 µg/doe, i.m.; Inducel-GnRH, Ovejero, León, Spain). At this moment dams were randomly allocated into three groups: *Ad libitum* feeding along the pregnancy (CONTROL; n=9), 50% restriction of their previous *ad libitum* intake throughout pregnancy (UNDERFED; n=12) or restricted only during the preimplantation period (D0-D7; ≈22% of the total pregnancy) followed by *ad libitum* feeding until the end of pregnancy (EARLY-UNDERFED; n=11). Food intake was also recorded weekly in all experimental groups during gestation.

Maternal blood sample collection

Evaluation of metabolic parameters and hormones was performed in five dams per experimental group. Blood was obtained weekly (D0, D7, D14, D21, D28) by ear-puncture. Blood was placed in tubes with Ethylenediaminetetraacetic acid (EDTA) as anticoagulant and centrifuged for 15 min at 1200 g to obtain ≈2 ml of plasma per dam. Concentrations of leptin were determined in a single analysis using the Multi-species Leptin RIA kit (Demeditec Diagnostics GmbH, Kiel, Germany). The assay sensitivity was 1.0 ng/ml and the intra-assay variation coefficient was 3.1%. Parameters relating to the glycemic (glucose and fructosamine) and lipid metabolisms (total cholesterol, high-density lipoproteins cholesterol [HDL-c], low-density lipoproteins cholesterol [LDL-c] and triglycerides) were measured on a clinical chemistry analyzer according to the manufacturer's instructions (Saturno 300 plus. Crony Instruments s.r.i., Rome, Italy).

Macroscopic study of the fetuses and placentas

Dams were weighed and euthanized (30 mg/kg, IV marginal ear vein administration; Dolethal, Madrid, Spain) at D28. The gravid uterus was removed by a medial laparotomy and subsequently weighed. Fetuses were dissected from their extraembryonic membranes and classified according to the following criteria: viable (with a correct morphology and weight for the gestational age), mummified (dead in uterus with signs of shrivel and drying) or fetal resorption (residual placental tissue attached to the endometrium) (Fig 1). The implantation rate was estimated as the ratio between total numbers of implanted fetuses and *corpora lutea* present in the ovaries. In viable fetuses, crown-rump length and bodyweight were determined by the use of scales and calipers

prior to decapitation, after which brain and liver tissue were weighed. Brain and liver to fetal weight-ratios and brain to liver weight-ratio were obtained as indexes for IUGR [18, 46]. Finally, placentas from viable fetuses (except those selected for histopathological study) were weighed and decidua and labyrinth sections were separated and characterized by weight, length, breadth and thickness with scales and calipers. Placental efficiency was calculated as fetal to placental weight-ratio [47, 48].

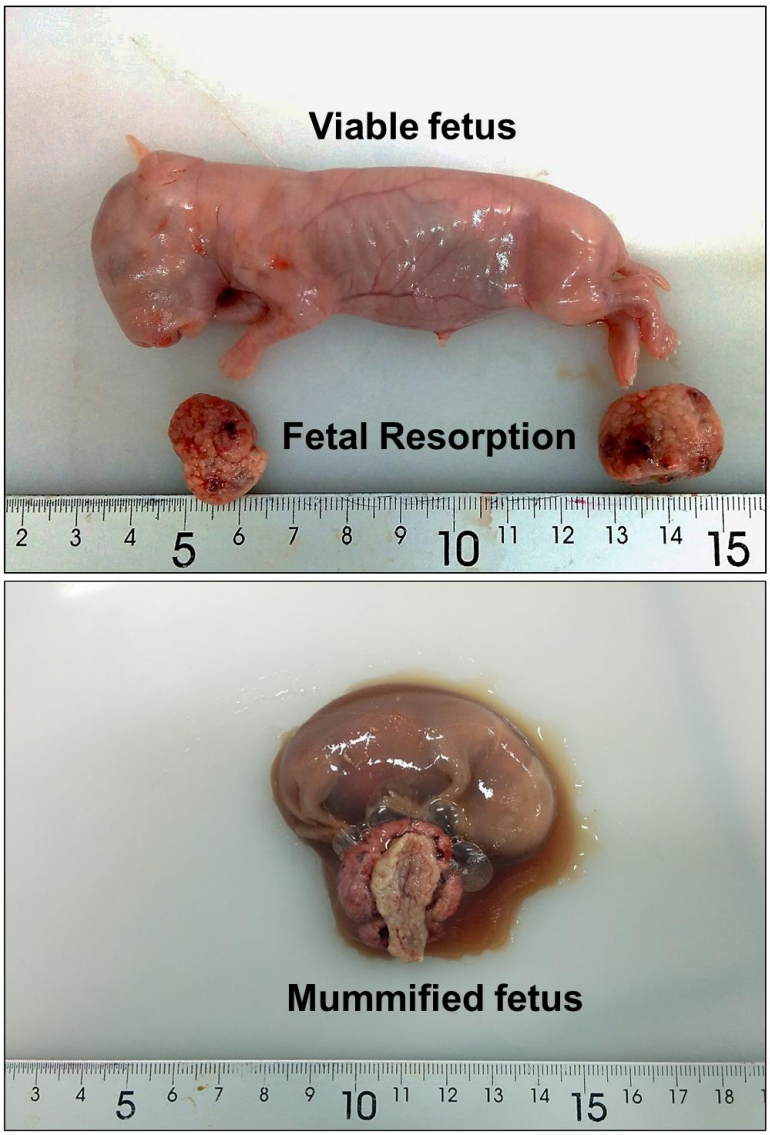


Fig 1. Macroscopic images of rabbit conceptus at D28 of pregnancy.

Microscopic study of placenta

The placentas closest to the left ovary were cut in half, fixed in 4% paraformaldehyde and switched to 70% ethanol the following day. Samples [CONTROL (n=8; one placenta was excluded for technical reasons), EARLY-UNDERFED (n=11), UNDERFED (n=12)] were embedded in paraffin and sectioned at 4 µm thickness with a semi-automated rotary microtome (Leica, Wetzlar, Germany).

a) Histopathological study of placenta

All samples were stained with hematoxylin and eosin and analyzed under a light microscope (Olympus BX40, Hamburg, Germany) by a trained pathologist blinded to the experimental groups. Stromal collapse of the labyrinth and decidual sclerosis of vascular channels were each graded and scored using the following criteria: unremarkable=0, mild=1, moderate=2 and severe=3. Other descriptive pathological features such as necrosis, mineralization and inflammatory infiltrates were recorded. The width of each placental layer (decidua, labyrinth and junctional zone) was analyzed by measuring five random fields of each layer under a 1.25x objective and obtaining the mean value.

b) Placental apoptosis study

Degree of apoptosis was determined using the ApopTag *in situ* apoptosis detection kit (Millipore Corp., San Francisco, USA) for terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL), according to the manufacturer's instructions and as previously described [49]. Sections were deparaffinized and treated with proteinase K (Roche Diagnostics GmbH, Mannheim, Germany). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate-buffered saline (PBS), and incubated with anti-digoxigenin conjugate in a humidified chamber at room temperature for 30 min. Sections were counterstained with methyl green. Ten random fields of each placental zone were photographed under a 20x objective and quantified using Image J software. The percentage of apoptotic cells was estimated as the number of TUNEL stained nuclei divided to the total number of total stained nuclei per zone × 100 [49].

To corroborate the results obtained from TUNEL assessment, we also performed Caspase-3 quantification. In brief, endogenous peroxidase activity was blocked by a 30 min treatment with 3% hydrogen peroxidase in absolute methanol. Nonspecific binding was blocked by incubating the sections in 5% normal goat serum (sc-2043, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated overnight with the primary antibody (1:25; caspase-3 antibody ab2171; Abcam, Cambridge, UK). After subsequent washes, sections were incubated with biotinylated anti-mouse secondary antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and subsequently with the avidin-biotin complex (ABC Vector Elite kit, Vector Laboratories, Burlingame, CA, USA). After chromogen incubation (Vector Nova RED substrate Kit for Peroxidase, Vector Laboratories, Burlingame, CA, USA), sections were counterstained with hematoxylin and analyzed under a light microscope (Leica DM IL, Wetzlar, Germany). The percentage of Caspase-3 positive immunostaining was determined as caspase-3 positive stain in a zone (pixels)/total surface of that zone (pixels) x 100.

Statistical Analysis

Statistical analysis was performed with SAS software (Statistical Analysis System Institute Inc., Cary, NC, USA). The effects of MFR in food intake, leptin and plasma metabolic parameters were evaluated by two-way analysis of variance (ANOVA). Maternal bodyweight and parameters related to conceptus development were analyzed by one-way ANOVA with maternal bodyweight at the beginning of the trial and number of fetuses per dam as covariates, respectively. If significant main effects were detected, T test (for parametric variables) or Kruskal-Wallis test (non-parametric variables) were used to compare averages among groups. Histological findings related to percentages were obtained by a Chi square test. All data were reported as mean±SEM and probabilities were considered significant at $P<0.05$.

RESULTS

Early-MFR induced maternal food intake compensation in later gestation and circumscribed changes in lipid metabolism without affecting bodyweight, leptin concentrations or glycemic profile.

Both undernourished groups had similar food intake during the 1st week of pregnancy and showed significant differences compared with the CONTROL group (Fig 2a). Once the restriction finished, EARLY-UNDERFED group significantly increased its food intake compared to the CONTROL group during the 2nd and 3rd weeks of pregnancy, but not during the last week of gestation, in which both groups significantly decreased their food intake (Fig 2a). These changes in dietary patterns were not associated to changes in the bodyweight of the dams at D28 calculated after retrieval of the pregnant uterus (Fig 2b). Furthermore, MFR did not affect either plasma leptin concentrations (Fig 2c), glucose or fructosamine levels (Fig 2d-e), but did alter the circulating lipid metabolites in early to mid-gestation (D0 to D14). In this regard, during the preimplantation period (D0 to D7) higher concentrations of triglycerides were found in the CONTROL group compared to both undernourished groups (Fig 2f). Meanwhile, plasma cholesterol concentrations were only significantly higher in the UNDERFED group on D14 *versus* the CONTROL and EARLY-UNDERFED groups (Fig 2g). On D7, plasma HDL-c concentrations were significantly reduced in both undernourished groups (Fig 2h) despite the fact that within the same period of gestation, LDL-c concentrations increased in these two groups respect CONTROL values (Fig 2i). Lastly, LDL-c concentrations remained higher only in the UNDERFED group until D14 compared to EARLY-UNDERFED and CONTROL groups (Fig 2i).

Fig 2. Effects of MFR on maternal food intake, bodyweight and plasma leptin, glucidic and lipid concentrations during pregnancy. *Number of pregnant dams per group employed for Fig. a) and b): CONTROL=9, EARLY-UNDERFED=11, UNDERFED=12; Number of pregnant dams per group employed for Fig. c) to i): CONTROL=5, EARLY-UNDERFED=5, UNDERFED=5. Statistical analyses were performed by ANOVA. Data presented as mean±SEM. Different superscripts indicate significant differences between groups (P<0.05).*

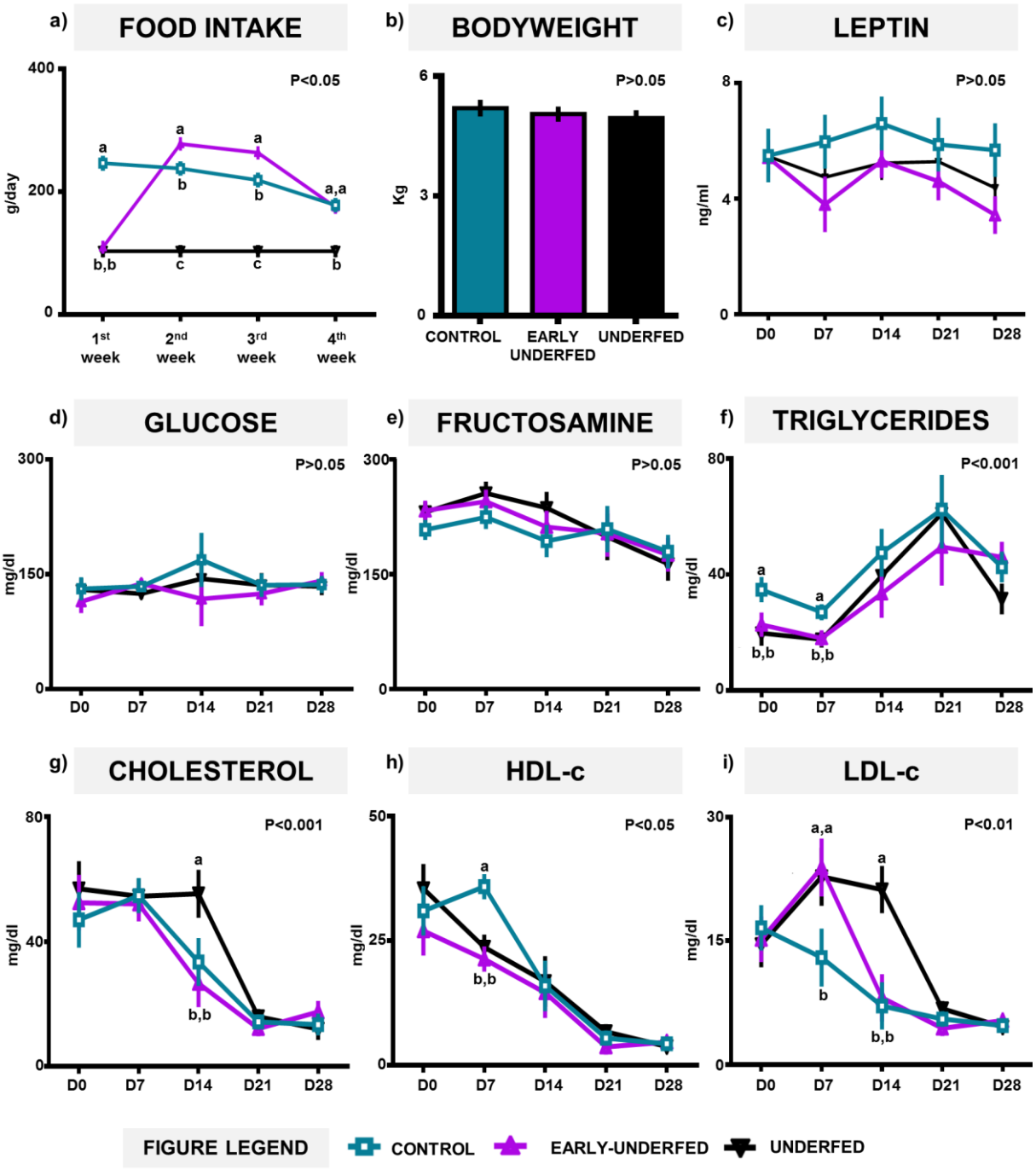


Table 1. Effects of MFR on conceptus development. *Number of pregnant dams per group: CONTROL=9, EARLY-UNDERFED=11, UNDERFED=12; Number of fetuses per group: CONTROL n=105, EARLY-UNDERFED n=126, UNDERFED n=117. Statistical analyses were performed by ANOVA. Data presented as mean±SEM. Different superscripts indicate significant differences between groups (P<0.01). NS non-significant differences.*

	CONTROL	EARLY-UNDERFED	UNDERFED	P
Litter size				
Gravid uterus (g)	762.96±52.82^{a}	757.65±47.78^{a}	572.19±45.74^{b}	<0.001
Total number of fetuses (n)	12.11±1.20	11.90±1.09	10.33±1.04	NS
Viable fetuses (n)	11.66±1.16	11.18±1.08	9.75±1.00	NS
Mummified fetuses (n)	0.22±0.19	0.36±0.17	0.16±0.16	NS
Fetal resorptions (n)	0.22±0.20	0.36±0.18	0.41±0.17	NS
Implantation rate (%)	88.69±9.06	92.27±8.01	93.37±7.63	NS
Fetuses				
Crown-Rump length (cm)	9.90±0.09^{a}	9.45±0.08^{b}	9.13±0.08^{c}	<0.001
Fetal weight (g)	42.10±0.67^{a}	41.10±0.60^{a}	35.67±0.63^{b}	<0.001
Head weight (g)	10.08±0.12^{a}	9.78±0.10^{a}	8.87±0.11^{b}	<0.001
Trunk weight (g)	31.48±0.54^{a}	30.89±0.48^{a}	26.25±0.51^{b}	<0.001
Brain weight (g)	1.03±0.01^{a}	1.06±0.01^{a}	0.96±0.01^{b}	<0.001
Brain ratio (%)	2.49±0.04^{a}	2.63±0.04^{b}	2.77±0.04^{c}	<0.001
Liver weight (g)	3.59±0.09^{a}	3.21±0.08^{b}	2.68±0.09^{c}	<0.001
Liver ratio (%)	8.42±0.15^{a}	7.60±0.14^{b}	7.41±0.14^{b}	<0.001
Brain:Liver ratio	0.31±0.01^{a}	0.37±0.01^{b}	0.39±0.01^{b}	<0.001
Placenta				
Placental Efficiency	7.0±0.12^{a}	6.76±0.11^{a}	7.51±0.12^{b}	<0.001
Total placenta weight (g)	5.99±0.15^{a}	5.91±0.14^{a}	4.67±0.15^{b}	<0.001
Decidua				
Weight (g)	1.56±0.05^{a}	1.52±0.04^{a}	1.22±0.04^{b}	<0.001
Length (cm)	3.55±0.07^{a}	3.72±0.07^{a}	3.24±0.07^{b}	<0.001
Breadth (cm)	1.43±0.04	1.38±0.03	1.37±0.04	NS
Thickness (cm)	0.40±0.02^{a}	0.37±0.01^{a}	0.31±0.01^{b}	<0.001
Labyrinth zone				
Weight (g)	4.12±0.12^{a}	3.88±0.11^{a}	3.13±0.12^{b}	<0.001
Length (cm)	3.66±0.05^{a}	3.57±0.05^{a}	3.28±0.05^{b}	<0.001
Breadth (cm)	2.68±0.06^{a}	2.60±0.06^{a}	2.35±0.06^{b}	<0.001
Thickness (cm)	0.52±0.02^{a}	0.49±0.02^{ab}	0.46±0.02^{b}	<0.01

MFR disrupted fetal growth trajectory but did not alter the number of viable fetuses at D28.

MFR reduced the weight of the gravid uterus in the UNDERFED group compared to the CONTROL and EARLY-UNDERFED groups, despite no differences in the number of fetuses per litter or in implantation rate (Table 1). Fetuses in the UNDERFED group showed the lowest crown-rump length and fetal weight (total, head and trunk) while the EARLY-UNDERFED group only showed a significant reduction of crown-rump length when compared to the CONTROL group. Brain and liver weight were significantly lower in the UNDERFED group when compared to the CONTROL and the EARLY-UNDERFED groups (Table 1). In the EARLY-UNDERFED group, liver weight was significantly lower than in the CONTROL group. However, no difference was found in the absolute weight of the brain between these two groups. The assessment of brain ratio showed a significant increase in both undernourished groups, while liver ratios were equally reduced in EARLY-UNDERFED and UNDERFED compared to the CONTROL. Thus, brain: liver weight ratio was higher in both restricted groups with respect to the CONTROL group.

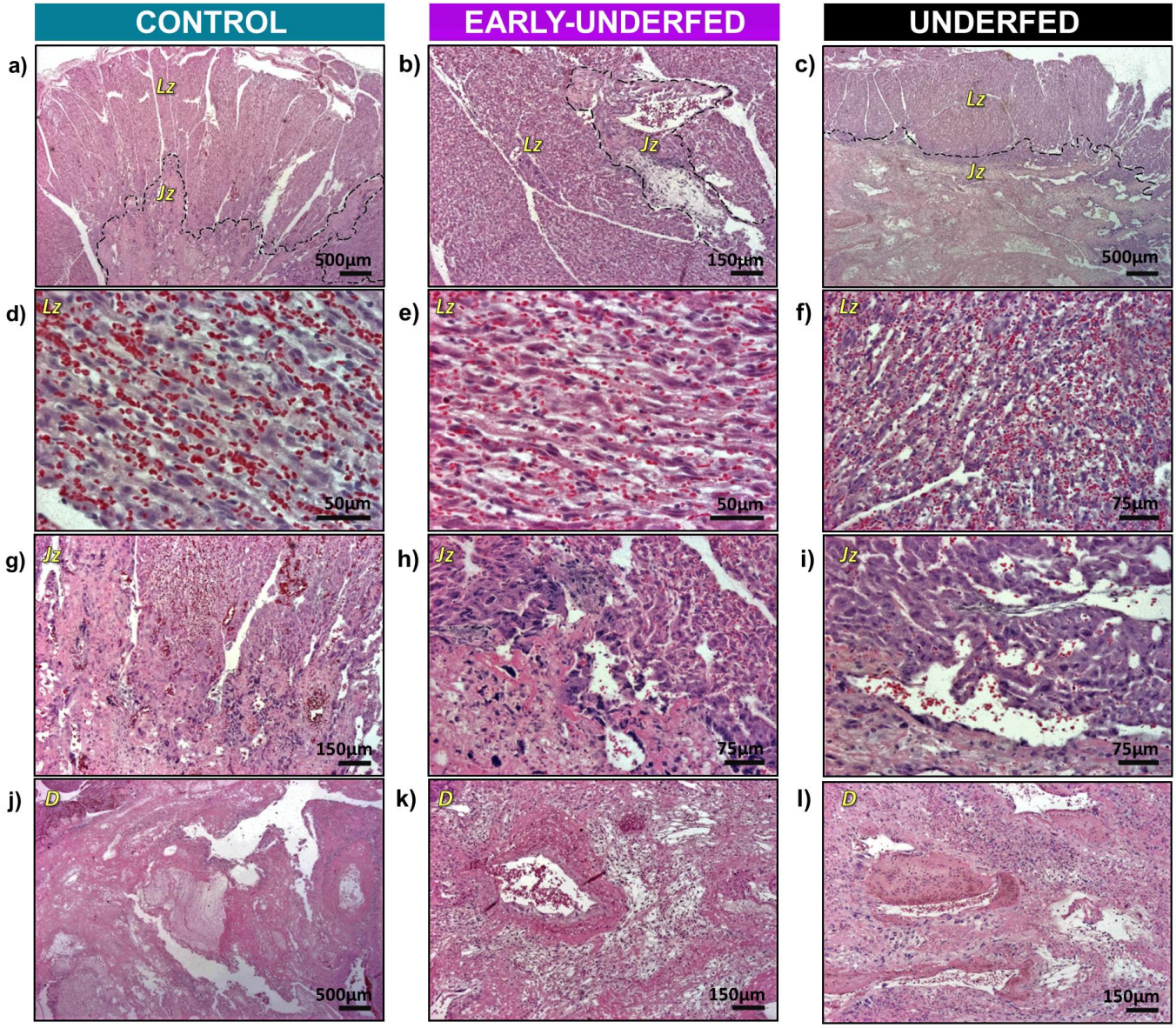
MFR altered placental development and resulted in histopathological changes and higher apoptotic levels.

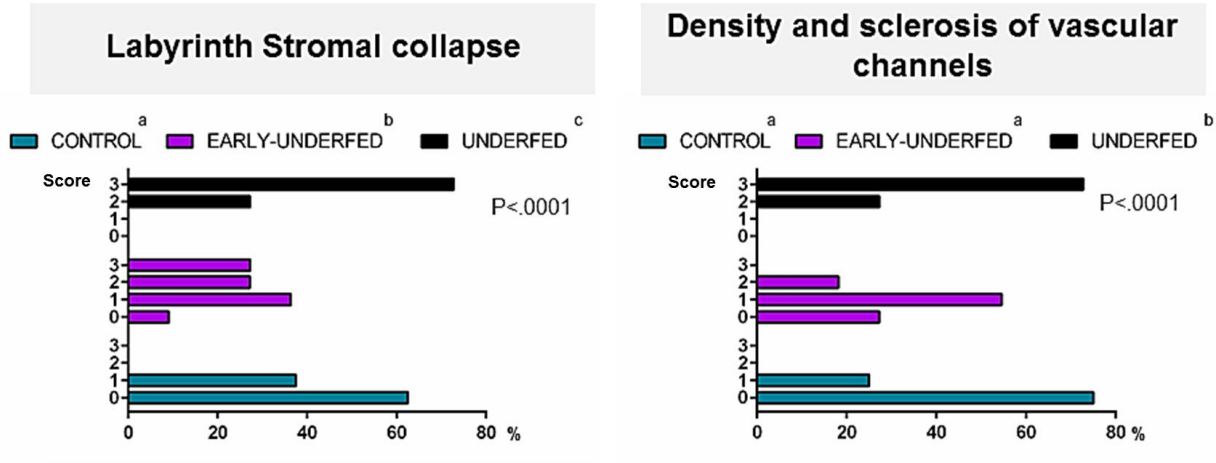
Placental development was altered in both undernourished groups with a more severe effect when MFR was carried out throughout gestation. MFR reduced placental weight only in the UNDERFED group although a compensatory placental efficiency was observed (measured as fetus-to-placenta weight-ratio; Table 1). Decidua and labyrinth sizes were reduced in this group except for the decidua breadth, which did not show significant differences among groups. In the EARLY-UNDERFED group, labyrinth thickness showed intermediate values between the CONTROL and UNDERFED groups. No other differences were observed between groups within the remaining macroscopic parameters measured.

Significant histological changes are summarized in Fig 3. The most notable changes in the placenta occurred in the UNDERFED group, which showed the lowest decidual width value (2.04 ± 0.32 mm) compared to the CONTROL (3.19 ± 0.32 mm) and the EARLY-UNDERFED groups (3.08 ± 0.29 mm) ($P<0.05$). Thus, the decidua of the UNDERFED group had fewer vessels, most of which contained hyalinized and sclerotic walls and lesser supporting stroma than the other groups (Fig 3). The labyrinth stroma in both restricted groups was significantly reduced in width

compared to the CONTROL group (2.46 ± 0.14, 2.04 ± 0.19 and 3.03 ± 0.20 mm, for EARLY-UNDERFED, UNDERFED and CONTROL groups, respectively; $P<0.05$). The reduced width corresponded with the stromal collapse of the labyrinth which contained lesser cellularity (vascular endothelium and trophoblasts) than the CONTROL group. Thus the UNDERFED group showed moderate to severe signs of collapse followed by the slightly less severe EARLY-UNDERFED group (Fig 3). Necrosis was observed in all experimental groups without significant differences (Fig 3). Mild mineralization was associated with the necrosis and was likewise insignificant between groups (Fig 3). The width of the junctional zone was similar between groups (CONTROL: 0.42 ± 0.08 mm; EARLY-UNDERFED: 0.35 ± 0.07 mm and UNDERFED: 0.30 ± 0.09 mm; $P>0.05$). Mild heterophilic inflammation was observed in the three layers of all the experimental groups and was within normal limits for this organ (Fig 3).

Concerning placental apoptotic rate, higher rates determined by TUNEL assay were found in both undernourished groups at the decidua and labyrinth zones (Fig 4a). At the junctional zone, only the UNDERFED group evidenced a significant higher rate when compared to the EARLY-UNDERFED and CONTROL groups (Fig 4a). Results from the assessment of caspase-3 levels (Fig 4b) were similar to those found in TUNEL assay in decidua and junctional zone. In contrast, the labyrinth showed higher rates of immunostaining in the UNDERFED group followed by the EARLY-UNDERFED group and compared to the CONTROL group.





	CONTROL	EARLY-UNDERFED	UNDERFED	P value
Multifocal labyrinth necrosis	12.50% (1/8)	27.27% (3/11)	25% (3/12)	NS
Mineralization within labyrinth	0% (0/8)	9.09% (1/11)	8.83% (1/12)	NS
Scattered heterophilic infiltration/three layers	62.50% (5/8)	54.54% (6/11)	50% (6/12)	NS

Fig 3. Histological findings of the rabbit placenta at D28 of pregnancy in CONTROL, EARLY-UNDERFED and UNDERFED groups. *Figures A to C: Labyrinth (Lz) and junctional (Jz) zones of the rabbit placenta in the three experimental groups. (D & E) CONTROL AND EARLY-UNDERFED groups. Densely cellular labyrinth with endothelium lined vascular channels and trophoblasts. (F) Labyrinth of the UNDERFED group with variably spaced endothelium lined vascular channels separated by collagen fibers (stromal collapse) and trophoblasts. Figures G to I: Junctional zone in the three experimental groups. (G) CONTROL group. Dense connective tissue matrix supporting stromal cells, trophoblasts and blood capillaries that extend into the labyrinth. (H) EARLY-UNDERFED group. Dense connective tissue containing stromal cells and trophoblasts. (I) UNDERFED group. Dense connective tissue slightly thinner and supporting fewer stromal cells and trophoblasts than the two other experimental groups. The overlaying labyrinth contains moderately spaced vascular channels with decreased cellularity. Figures J to L: Decidua (D) in the three experimental groups. (J) CONTROL GROUP. Normal decidua with large vascular sinuses, stroma, fibrin and necrosis. (K) EARLY-UNDERFED group. Large vessel within the decidua surrounded by abundant edematous stroma. (L) UNDERFED group. Markedly thinned decidua layer with spaced vessels containing hyalinised walls, a thrombus, and surrounded by dense stromal collagen. Number of placentas per group: CONTROL=8, EARLY-UNDERFED=11, UNDERFED=12; Statistical analyses were performed by Chi square test. In the charts and table different superscripts indicate significant differences between groups (P<0.0001), NS non-significant differences.*

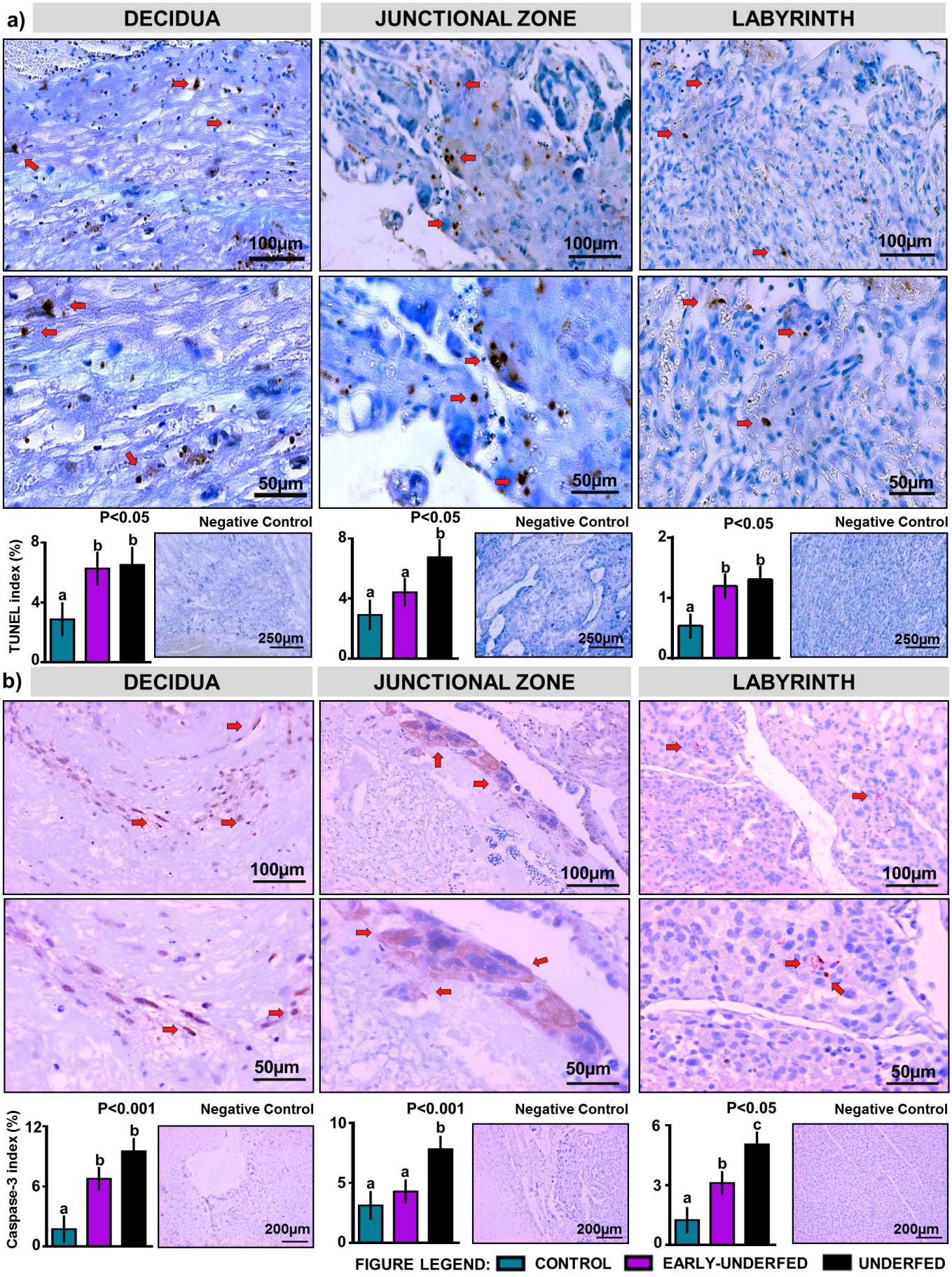

a)
DECIDUA
JUNCTIONAL ZONE
LABYRINTH
100µm
50µm
P<0.05
Negative Control
TUNEL index (%)
250µm
b)
P<0.001
Caspase-3 index (%)
200µm
FIGURE LEGEND: CONTROL EARLY-UNDERFED UNDERFED

Fig 4. Apoptosis assessments of the rabbit placenta at D28 of pregnancy. (a) Apoptosis quantified by TUNEL (positive staining) in decidua, junctional zone and labyrinth. (b) Localization and percentage of Caspase-3 (positive immunostaining) in decidua, junctional zone and labyrinth. *Number of placentas per group: CONTROL=8, EARLY-UNDERFED=11, UNDERFED=12; Statistical analyses were performed by ANOVA. Data presented as mean±SEM. Different superscripts in the charts indicate significant differences between groups (P<0.05).*

DISCUSSION

The current study shows that MFR applied to pregnant rabbits, despite the absence of significant changes in maternal bodyweight, plasma leptin concentration or glucidic metabolites, affects maternal lipid concentrations during early to mid-gestation but not during late gestation, when fetal growth is exponential. MFR applied only during the preimplantational period did not result in low fetal weight, but was enough to disrupt crown-rump length and impair organogenesis. Consistent with these findings, severe alterations on placental and fetal development can be observed when MFR is maintained throughout gestation (Fig 5).

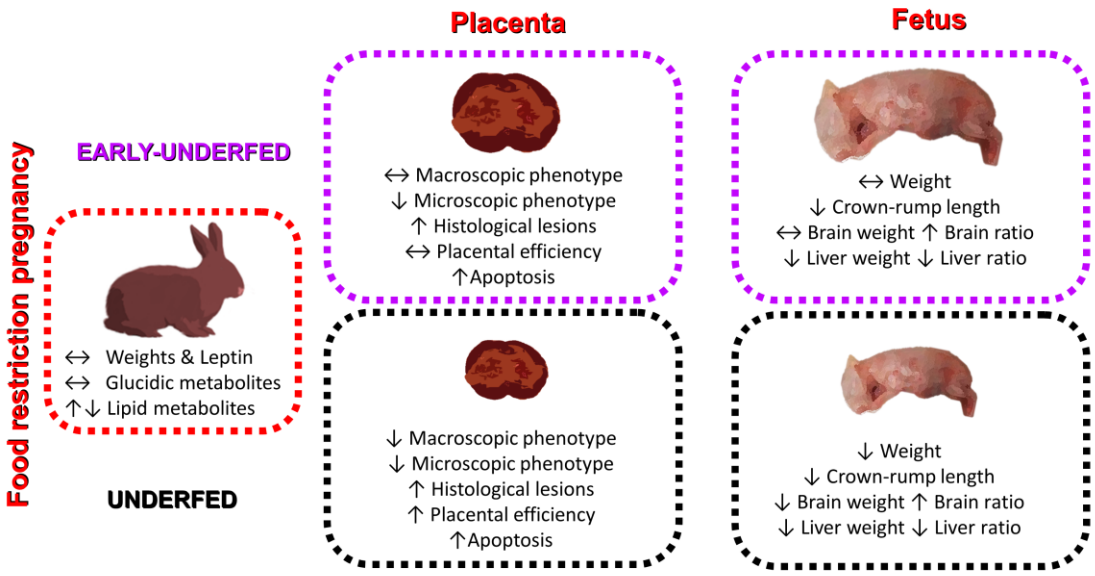


Fig 5. Summary illustration showing the effects of MFR in the EARLY-UNDERFED and UNDERFED groups on placental and fetal outcome.

The lack of effects of MFR on maternal bodyweight found in the current study supports previous studies in rabbits [30, 38, 41] and differs from other animal species in which the application of MFR resulted in maternal bodyweight loss [rodents: 50-53; sheep: 54, 55; primates: 56, 57]. These differences between species and experimental studies may be related to feeding management (*ad libitum* feeding *vs* maintenance requirements), fat stores prior to the experimental phase, or may be as well associated to reproductive strategies adopted by the mothers [58]. In this sense, undernourished dams had similar implantation rates and litter sizes compared to the CONTROL group. These results may suggest that undernourished mothers do not invest more than indispensable in their offspring growth, as a preventive strategy for future gestations and for their own energetic balance [58]. Despite the fact that offspring growth was affected, undernourished females could have accumulated resources to deal with the lactation, to guarantee a better offspring feeding, postnatal survival and growth and therefore ensure the transmission of maternal genes to future generations [58].

Concomitantly, plasma leptin concentrations did not decline in any of the groups in our study, opposite to previous works in rabbit [37], mouse [51, 59] and sheep [55]. We hypothesize that the absence of differences in our study may be explained by different facts. At first glance, the most obvious reason could be related to the number of animals sampled. However, sample size was calculated according to the study of Menchetti et al. [37], which found variations in leptin profile using a similar number of pregnant rabbits. Other explanations may involve an increased production of leptin either by maternal, placental or fetal tissues [52, 60]. This hypothesis may reinforce the idea that despite the restriction, food amount could be enough to satisfy maternal and fetal requirements. Expression of leptin-RNA in maternal adipose and fetoplacental tissues should corroborate this idea. Lastly, leptin concentrations could be influenced by hormonal milieu, specially insulin and/or glucose [61], estrogens [62] or glucocorticoids, which have been shown to induce leptin expression [63].

In contrast, maternal plasma lipids changed but only in early to mid-gestation. Such period of pregnancy can be considered as an anabolic state, due to the low requirements of the developing offspring [64, 65]. In contrast, we did not observe any variations in lipid profile during the last third of gestation, a period of accelerated breakdown of fat depots to satisfy fetal demands [66, 67]. In this regard, plasma cholesterol and LDL-c concentrations increased in the undernourished groups during the period comprising D7 to D14 of pregnancy, suggesting a certain level of fat

mobilization may be needed to deal with the implantation process [68], yolk sac formation and the establishment of the chorio-allantoic placental circulation [69]. In contrast, HDL-c concentrations raised in the CONTROL group to return excess of cholesterol from peripheral tissues back to the liver in response to an adequate level of resources [70]. Furthermore, maternal triglycerides concentrations were lower in both undernourished groups which may be a mechanism to increase deposition of fat in maternal adipose tissue for lactation [71]. Overall, these findings may reinforce, once more, the aforementioned strategies adopted by the mothers to constrain allocation of resources during late gestation on behalf of themselves and future lactation.

Following this argument, the placenta is central to this "tug of war" over nutrient allocation as it is the surface area for exchange between mother and fetus [58; 72]. Experiments based on genetic and dietary manipulations have demonstrated that the placenta interprets fetal and maternal interests, adapting its phenotype and function according to resource availability [72-74]. An example of these fascinating adaptations is that undernourished placentas can overcome MFR and maintain or even increase their nutrient transfer capacity to help maintain fetal growth [50]. In our study, placental weight was reduced in the UNDERFED group. Such finding supports the hypothesis that the preimplantational period may be a critical timing for placental establishment, since the same level of MFR applied after implantation (D9 of gestation in rabbits) did not reduce placental weight [31].

Conversely, no gross macroscopic differences were found in the EARLY-UNDERFED, reinforcing previous findings in sheep that were only restricted during early-mid gestation, and then returned to normal feeding [75]. Interestingly, the microscopic assessment of placental thickness showed a significant reduction in labyrinth expansion in both undernourished groups, which supports previous work [35]. The progressive reduction in the surface area of the labyrinth may affect placental nutrient transporters and contribute to the poor fetal outcome. In rats, MFR by 50% downregulated *GLUT3* transporter expression, resulting in offspring with IUGR [76]. Furthermore, in a primate model of MFR [77], expression of glucose and amino acid transporters (*GLUT1*, *TAUT*, *SNAT2*, *LAT1*, and *LAT2*) were reduced as well as placental and fetal weights. Future work should evaluate such findings in our rabbit model of MFR and its connection with IUGR.

The reduction in the size of the decidua only observed in the UNDERFED group could be associated to two different processes. Firstly, a reduced trophoblast invasion could have impaired the correct remodeling process of the spiral arteries. Thus, the high incidence of sclerotic processes in the decidual vascular bed only observed in the UNDERFED group could be linked to the high circulating levels of cholesterol observed in this group on D14. In this sense, lipid peroxides and oxygen radicals can alter endothelial cells, resulting in fibrin deposition in the vessel walls [67]. Consequently, the arterial sinuses system in the rabbit, which can be identified within this period of gestation [78] and is responsible to retain maternal blood flow and then supply it to fetal area could be altered, resulting in IUGR. On the other hand, the reduction in decidua size could be a mechanism of these placentas to support fetal demands for growth by depletion of their glycogen reserves allocated in the small uninucleated cells of this placental section [79], and therefore may explain the increase in placental efficiency and the reduction in weight. However, this last effect remains speculative in this study; further investigations should corroborate this hypothesis and determine placental glycogen storage in restricted placentas of rabbit dams, as glycogen reserves in the murine placenta are an important source of glucose in the final stages of gestation [80]. However, what we have demonstrated is that imbalanced diets during pregnancy generate higher rates of apoptosis that could have affected placental development and function. Our study sets the bases on placental apoptosis in rabbit placenta at term and is in line with the range of degree of apoptosis reported in previous studies of MFR [49] and placental localization in mouse [81], showing higher rates of apoptosis in the decidua with respect to the other zones. Thus, this study has corroborated that apoptosis in placenta tissue is a biological phenomenon and could be a mechanism of placental remodeling [82], that may be associated to physiological degenerative mechanisms of the rabbit placenta near term [79, 83]. However, MFR significantly increases their rates, which may have resulted in placental insufficiency and therefore in IUGR.

As expected, UNDERFED fetuses evidenced the clearest signs of IUGR, with reductions in fetal size (weight and length) and organs (brain and liver). This reduction in fetal weight induced by MFR has been previously demonstrated in other animals such as rodents [53, 84] and sheep [85]. Our data have revealed that MFR applied only during the preimplantational period did not reduce fetal weight despite that both fetal size and organogenesis were affected. The results obtained by the ratios assessments, suggest certain level of asymmetric IUGR in both MFR groups;

reinforcing the innate mechanisms of vasodilation and blood shunting of the fetus to safeguard the growth of key organs like the brain (process known as "brain-sparing effect"), even at the expense of the growth of other tissues (*e.g.* the liver, thymus or skeletal muscle) [31, 86, 87]. The possible postnatal consequences of these prenatal adaptations induced by MFR remain unclear in our model. In previous work, rabbit offspring developed in a MFR environment evidenced altered eating, drinking and locomotor behaviors [88], which may suggest changes in brain network organization. In humans, IUGR infants with brain sparing, showed worse neurodevelopmental and behavioural outcomes than those without signs of such mechanism [87]. In rodents, as recently reviewed by Sferruzzi-Perri and Camm [12], MFR can induce early modifications in cerebral structure, contributing to late-onset diseases in the offspring. Moreover, the cost of sparing the brain reduced liver mass in both undernourished groups, which is highly important for neonatal life, as it enables fat deposition and acts as a source of growth factors and glycogen. In addition, this prenatal reduction of liver mass along with the possible hepatic gene dysregulation already reported in IUGR rats [89] could predispose the offspring to suffering from metabolic diseases in adulthood, such as obesity, insulin resistance and type-2 diabetes [90-92]. These adaptations are likely to have implications for subsequent postnatal growth and the quality of the meat as recently reviewed by Chavatte-Palmer [2, 8]. In sheep, 50% MFR resulted in offspring with increased fat deposition and altered glucose metabolism [93]. However, the most recent studies performed in rabbits, have not found differences in meat quality parameters restricting dams by 50% [94] or 75% [95].

In conclusion, the present study has helped increase awareness of the effects of MFR in gestation. The results of the present study suggest that MFR may induce strategic decisions in the mothers to safeguard their bodyweight and metabolic status at the expense of reductions in the growth of their litters. These maternal decisions are associated with moderate changes in lipid metabolism in circumscribed periods of the pregnancy (mainly during embryo development and early placental formation), but not in leptin secretion or glucidic metabolites. Thus, MFR impairs placenta development and enhances apoptotic processes, which ultimately could reduce its functionality and lastly induce IUGR. Fetuses will be reduced in size, and organogenesis can be impaired, even if the exposure only occurs during the preimplantation period. Therefore, these results should be taken into consideration when these kind of nutritional strategies were applied in livestock animals, as no clear evidence are found in the mothers, but the progeny can be affected. Furthermore, this study reinforces the use of rabbits as valid biomedical models in the

perinatal field. Additional studies are needed to determine the possible consequences induced by the MFR in the offspring fitness.

Conflict of interest

There is no conflict of interest that would prejudice the information offered in the paper, excepting that AGB is a PLOS ONE Editorial Board member. However, this does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Author Contributions

Conceived and designed the experiments: JL-T, MA-A, PGR. Performed the experiments: JL-T, MA-A, MAJ-M, RMG-G, MR, PLLG, RB-P, AG-B, PGR. Analyzed the data: JL-T, MA-A, RB-P, AG-B, PGR. Wrote the paper: JL-T, MA-A, MAJ-M, AG-B, PGR. Revised the manuscript: JL-T, MA-A, MAJ-M, RMG-G, MR, PLLG, RB-P, AG-B, PGR.

References

[1] Wu G, Bazer FW, Wallace JM, Spencer TE. Board-invited review: intrauterine growth retardation: implications for the animal sciences. J Anim Sci. 2006;84(9):2316-37.

[2] Chavatte-Palmer P, Tarrade A, Kiefer H, Duranthon V, Jammes H. Breeding animals for quality products: not only genetics. Reprod Fertil Dev. 2016;28(1-2):94-111.

[3] Albu A, Anca A, Horhoianu V, Horhoianu I. Predictive factors for intrauterine growth restriction. J Med Life. 2014;7(2):165-171.

[4] Barker DJ. The developmental origins of adult disease. J Am Coll Nutr. 2004;23(6 Suppl):588s-95s.

[5] Barker DJ. The developmental origins of well-being. Philos Trans R Soc Lond B Biol Sci. 2004;359(1449):1359-66.

[6] de Rooij SR, Wouters H, Yonker JE, Painter RC, Roseboom TJ. Prenatal undernutrition and cognitive function in late adulthood. PNAS. 2010;107(39):16881-6.

[7] Campos PH, Silva BA, Donzele JL, Oliveira RF, Knol EF. Effects of sow nutrition during gestation on within-litter birth weight variation: a review. Animal. 6;2012:797-806.

[8] Chavatte-Palmer P, Richard C, Peugnet P, Robles M, Rousseau-Ralliard D, Tarrade A. The developmental origins of health and disease: importance for animal production. Anim Reprod. 2015;12:505-20.

[9] Manal AF, Tony MA, Ezzo OH. Feed restriction of pregnant nulliparous rabbit does: consequences on reproductive performance and maternal behaviour. Anim Reprod Sc. 2010;120(1):179-186.

[10] Rommers JM, Meijerhof R, Noordhuizen JP, Kemp B. The effect of level of feeding in early gestation on reproductive success in young rabbit does. Anim Reprod Sci. 2004;81(1-2):151-8.

[11] Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Development. 2000;127(19):4195-4202.

[12] Sferruzzi-Perri AN, Camm EJ. The Programming Power of the Placenta. Front Physiol. 2016;7:33.

[13] Belkacemi L, Nelson DM, Desai M, Ross MG. Maternal undernutrition influences placental-fetal development. Biol Reprod. 2010; 83(3):325-331.

[14] Schröder HJ. Models of fetal growth restriction. EJOG. 2003;110:S29-S39.

[15] Allain D, Renieri C. Genetics of fibre production and fleece characteristics in small ruminants, Angora rabbit and South American camelids. Animal. 2010;4(9):1472-81.

[16] Dalle Zotte A. Rabbit farming for meat purposes. Animal Frontiers. 2014;4(4):62-67.

[17] Eixarch E, Hernandez-Andrade E, Crispi F, Illa M, Torre I, Figueras F, et al. Impact on fetal mortality and cardiovascular Doppler of selective ligature of uteroplacental vessels compared with undernutrition in a rabbit model of intrauterine growth restriction. Placenta. 2011;32(4):304-9.

[18] Harel S, Yavin E, Tomer A, Barak Y, Binderman I. Brain: body ratio and conceptional age in vascular-induced intrauterine growth retarded rabbits. Brain Dev. 1985;7(6):575-9.

[19] Bassan H, Trejo LL, Kariv N, Bassan M, Berger E, Fattal A, et al. Experimental intrauterine growth retardation alters renal development. Pediatr Nephrol. 2000;15(3-4):192-5.

[20] Eixarch E, Figueras F, Hernandez-Andrade E, Crispi F, Nadal A, Torre I, et al. An experimental model of fetal growth restriction based on selective ligature of uteroplacental vessels in the pregnant rabbit. Fetal Diagn Ther. 2009;26(4):203-11.

[21] Bassan H, Kidron D, Bassan M, Rotstein M, Kariv N, Giladi E, et al. The effects of vascular intrauterine growth retardation on cortical astrocytes. J Matern Fetal Neonatal Med. 2010;23:595-600.

[22] Eixarch E, Batalle D, Illa M, Munoz-Moreno E, Arbat-Plana A, Amat-Roldan I, et al. Neonatal neurobehavior and diffusion MRI changes in brain reorganization due to intrauterine growth restriction in a rabbit model. PLoS One. 2012;7(2):e31497.

[23] Gonzalez-Tendero A, Torre I, Garcia-Canadilla P, Crispi F, Garcia-Garcia F, Dopazo J, et al. Intrauterine growth restriction is associated with cardiac ultrastructural and gene expression changes related to the energetic metabolism in a rabbit model. Am J Physiol Heart Circ Physiol. 2013;305(12):H1752-60.

[24] Illa M, Eixarch E, Batalle D, Arbat-Plana A, Munoz-Moreno E, Figueras F, et al. Long-term functional outcomes and correlation with regional brain connectivity by MRI diffusion tractography metrics in a near-term rabbit model of intrauterine growth restriction. PLoS One. 2013;8(10):e76453.

[25] Torre I, Gonzalez-Tendero A, Garcia-Canadilla P, Crispi F, Garcia-Garcia F, Bijnens B, et al. Permanent cardiac sarcomere changes in a rabbit model of intrauterine growth restriction. PLoS One. 2014;9(11):e113067.

[26] Figueroa H, Cifuentes J, Lozano M, Alvarado C, Cabezas C, Eixarch E, et al. Nitric oxide synthase and changes in oxidative stress levels in embryonic kidney observed in a rabbit model of intrauterine growth restriction. Prenat Diagn. 2016.

[27] Thakur A, Sase M, Lee JJ, Thakur V, Buchmiller TL. Effect of dexamethasone on insulin-like growth factor-1 expression in a rabbit model of growth retardation. J Pediatr Surg. 2000;35(6):898-904.

[28] Lecarpentier E, Morel O, Tarrade A, Dahirel M, Bonneau M, Gayat E, et al. Quantification of utero-placental vascularization in a rabbit model of IUGR with three-dimensional power Doppler angiography. Placenta. 2012;33(10):769-75.

[29] Tarrade A, Lecarpentier E, Gil S, Morel O, Zahr N, Dahirel M, et al. Analysis of placental vascularization in a pharmacological rabbit model of IUGR induced by L-NAME, a nitric oxide synthase inhibitor. Placenta. 2014;35(4):254-9.

[30] Lopez-Tello J, Barbero A, Gonzalez-Bulnes A, Astiz S, Rodriguez M, Formoso-Rafferty N, et al. Characterization of early changes in fetoplacental hemodynamics in a diet-induced rabbit model of IUGR. J Dev Orig Health Dis. 2015;6(5):454-61.

[31] Lopez-Tello J, Arias-Alvarez M, Jimenez-Martinez MA, Barbero-Fernandez A, Garcia-Garcia RM, Rodriguez M, et al. The effects of sildenafil citrate on feto?placental development and haemodynamics in a rabbit model of intrauterine growth restriction. Reprod Fertil Dev. 2016.

[32] Fischer B, Chavatte-Palmer P, Viebahn C, Navarrete Santos A, Duranthon V. Rabbit as a reproductive model for human health. Reproduction. 2012;144(1):1-10.

[33] Derrick M, Drobyshevsky A, Ji X, Chen L, Yang Y, Ji H, et al. Hypoxia-ischemia causes persistent movement deficits in a perinatal rabbit model of cerebral palsy: assessed by a new swim test. Int J Dev Neurosci. 2009;27(6):549-57.

[34] Polisca A, Scotti L, Orlandi R, Brecchia G, Boiti, C. Doppler evaluation of maternal and fetal vessels during normal gestation in rabbits. Theriogenology. 2010;73(3): 358-366.

[35] Matsuoka T, Mizoguchi Y, Haneda R, Otsuka E, Mizuguchi H, Fukuda K, et al. Effects of restricted feeding on fetal and placental development in pregnant rabbits. J Toxicol Sci. 2012;37(1):207-14.

[36] Nafeaa A, Ahmed SA, Fat Hallah S. Effect of Feed Restriction during Pregnancy on Performance and Productivity of New Zealand White Rabbit Does. Vet Med Int. 2011;2011:839737.

[37] Menchetti L, Brecchia G, Canali C, Cardinali R, Polisca A, Zerani M, et al. Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming. Res Vet Sci. 2015;98:7-12.

[38] Menchetti L, Brecchia G, Cardinali R, Polisca A, Boiti C. Food restriction during pregnancy: effects on body condition and productive performance of primiparous rabbit does. World Rabbit Sci. 2015;23:1-8.

[39] Cappon GD, Fleeman TL, Chapin RE, Hurtt ME. Effects of feed restriction during organogenesis on embryo-fetal development in rabbit. Birth Defects Res B Dev Reprod Toxicol. 2005;74(5):424-30.

[40] Petrere JA, Rohn WR, Grantham LE, Anderson JA. Food restriction during organogenesis in rabbits: effects on reproduction and the offspring. Fundam Appl Toxicol. 1993;21(4):517-22.

[41] Matsuzawa T, Nakata M, Goto I, Tsushima M. Dietary deprivation induces fetal loss and abortion in rabbits. Toxicology. 1981;22(3):255-9.

[42] Reynolds SRM. The relation of hydrostatic conditions in the uterus to the size and shape of the conceptus during pregnancy: A concept of uterine accommodation. Anat Rec. 1946;95(3):283-296.

[43] Schulz LC, Schlitt JM, Caesar G, Pennington KA. Leptin and the placental response to maternal food restriction during early pregnancy in mice. Biol Reprod. 2012;87(5):120.

[44] Dwyer CM, Madgwick AJ, Ward SS, Stickland NC. Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea-pig. Reprod Fertil Dev. 1995;7(5):1285-92.

[45] Jones AP, Friedman MI. Obesity and adipocyte abnormalities in offspring of rats undernourished during pregnancy. Science. 1982;215(4539):1518-9.

[46] Cox P, Marton T. Pathological assessment of intrauterine growth restriction. Best Pract Res Clin Obstet Gynaecol. 2009;23(6):751-64.

[47] Wilson ME, Ford SP. Comparative aspects of placental efficiency. Reprod Suppl. 2001;58:223-32.

[48] Fowden AL, Sferruzzi-Perri AN, Coan PM, Constancia M, Burton GJ. Placental efficiency and adaptation: endocrine regulation. J Physiol. 2009;587(Pt 14):3459-72.

[49] Belkacemi L, Chen CH, Ross MG, Desai M. Increased placental apoptosis in maternal food restricted gestations: role of the Fas pathway. Placenta.2009;30:739-51.

[50] Coan PM, Vaughan OR, Sekita Y, Finn SL, Burton GJ, Constancia M, et al. Adaptations in placental phenotype support fetal growth during undernutrition of pregnant mice. J Physiol. 2010;588(Pt 3):527-38.

[51] Sferruzzi-Perri AN, Vaughan OR, Coan PM, Suciu MC, Darbyshire R, Constancia M, et al. Placental-specific Igf2 deficiency alters developmental adaptations to undernutrition in mice. Endocrinology. 2011;152(8):3202-12.

[52] Jelks A, Belkacemi L, Han G, Chong WL, Ross MG, Desai M. Paradoxical increase in maternal plasma leptin levels in food-restricted gestation: contribution by placental and adipose tissue. Reprod Sci. 2009;16(7):665-75.

[53] Kind KL, Simonetta G, Clifton PM, Robinson JS, Owens JA. Effect of maternal feed restriction on blood pressure in the adult guinea pig. Exp Physiol. 2002;87(4):469-77.

[54] Satterfield MC, Bazer FW, Spencer TE, Wu G. Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. J Nutr. 2010;140(2):251-8.

[55] Debus N, Chavatte-Palmer P, Viudes G, Camous S, Rosefort A, Hassoun P. Maternal periconceptional undernutrition in Merinos d'Arles sheep: 1. Effects on pregnancy and reproduction results of dams and offspring growth performances. Theriogenology. 2012;77(7):1453-65.

[56] Rodriguez JS, Bartlett TQ, Keenan KE, Nathanielsz PW, Nijland MJ. Sex-dependent cognitive performance in baboon offspring following maternal caloric restriction in pregnancy and lactation. Reprod Sci. 2012;19(5):493-504.

[57] Antonow-Schlorke I, Schwab M, Cox LA, Li C, Stuchlik K, Witte OW, et al. Vulnerability of the fetal primate brain to moderate reduction in maternal global nutrient availability. PNAS. 2011;108(7):3011-6.

[58] Fowden AL, Moore T. Maternal-fetal resource allocation: co-operation and conflict. Placenta. 2012;33 Suppl 2:e11-5.

[59] Schlitt JM, Schulz LC. The source of leptin, but not leptin depletion in response to food restriction, changes during early pregnancy in mice. Endocrine. 2012;41(2):227-35.

[60] Kirat D, Hamid, NEA, Mohamed WE, Hamada M, Shalaby SI. Leptin Gene Expression in Rabbits During Pregnancy and Fetal Life. IJB. 2015;7(2): 113.

[61] Boden G, Chen X, Mozzoli M, Ryan I. Effect of fasting on serum leptin in normal human subjects. J Clin Endocrinol Metab. 1996;81(9):3419-23.

[62] Henson MC, Castracane VD. Leptin in pregnancy: an update. Biol Reprod. 2006;74(2):218-29.

[63] De Vos P, Saladin R, Auwerx J, Staels B. Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. J Biol Chem. 1995;270(27):15958-61.

[64] Lain KY, Catalano PM. Metabolic changes in pregnancy. Clin Obstet Gynecol. 2007;50(4):938-48.

[65] Musial B, Fernandez-Twinn DS, Vaughan OR, Ozanne SE, Voshol P, Sferruzzi-Perri AN, et al. Proximity to Delivery Alters Insulin Sensitivity and Glucose Metabolism in Pregnant Mice. Diabetes. 2016;65(4):851-60.

[66] Herrera E. Lipid metabolism in pregnancy and its consequences in the fetus and newborn. Endocrine. 2002;19(1):43-55.

[67] Chandi A, Sirohiwal D, Malik R. Association of early maternal hypertriglyceridemia with pregnancy-induced hypertension. Arch Gynecol Obstet. 2015;292(5):1135-43.

[68] Hoffman LH, Breinan DR, Blaeuer GL. The rabbit as a model for implantation: in vivo and in vitro studies. In *Embryo Implantation.* Springer New York. 1999;151-160.

[69] Carney EW, Scialli AR, Watson RE, DeSesso JM. Mechanisms regulating toxicant disposition to the embryo during early pregnancy: an interspecies comparison. Birth Defects Res C Embryo Today. 2004;72(4):345-60.

[70] Kondo A, Li J, Manabe M, Saito K, Kanno T, Maekawa M. Relationship between high-density lipoprotein-cholesterol and malondialdehyde-modified low-density lipoprotein concentrations. J Atheroscler Thromb. 2003;10(2):72-8.

[71] Lippi G, Albiero A, Montagnana M, Salvagno GL, Scevarolli S, Franchi M, et al. Lipid and lipoprotein profile in physiological pregnancy. Clin Lab. 2007;53(3-4):173-7.

[72] Fowden AL, Ward JW, Wooding FP, Forhead AJ, Constancia M. Programming placental nutrient transport capacity. J Physiol. 2006;572(Pt 1):5-15.

[73] Sandovici I, Hoelle K, Angiolini E, Constancia M. Placental adaptations to the maternal-fetal environment: implications for fetal growth and developmental programming. Reprod Biomed Online. 2012;25(1):68-89.

[74] Sferruzzi-Perri AN, Lopez-Tello J, Fowden AL, Constancia M. Maternal and fetal genomes interplay through phosphoinositol 3-kinase(PI3K)-p110α signalling to modify placental resource allocation. PNAS. 2016;113(40):11255-11260.

[75] Clarke L, Heasman L, Juniper DT, Symonds ME. Maternal nutrition in early-mid gestation and placental size in sheep. Br J Nutr. 1998;79(4):359-64.

[76] Lesage J, Hahn D, Leonhardt M, Blondeau B, Breant B, Dupouy JP. Maternal undernutrition during late gestation-induced intrauterine growth restriction in the rat is associated with impaired placental GLUT3 expression, but does not correlate with endogenous corticosterone levels. J Endocrinol. 2002;174(1):37-43.

[77] Kavitha JV, Rosario FJ, Nijland MJ, McDonald TJ, Wu G, Kanai Y, et al. Down-regulation of placental mTOR, insulin/IGF-I signaling, and nutrient transporters in response to maternal nutrient restriction in the baboon. Faseb j. 2014;28(3):1294-305.

[78] Khan H, Kusakabe KT, Wakitani S, Hiyama M, Takeshita A, Kiso Y. Expression and localization of NO synthase isoenzymes (iNOS and eNOS) in development of the rabbit placenta. J Reprod Dev. 2012;58(2):231-6.

[79] Samuel CA, Jack PM, Nathanielsz PW. Ultrastructural studies of the rabbit placenta in the last third of gestation. J Reprod Fertil. 1975;45(1):9-14.

[80] Coan PM, Conroy N, Burton GJ, Ferguson-Smith AC. Origin and characteristics of glycogen cells in the developing murine placenta. Dev Dyn. 2006;235(12):3280-94.

[81] Mu J, Kanzaki T, Tomimatsu T, Fukuda H, Wasada K, Fujii E, et al. Expression of apoptosis in placentae from mice lacking the prostaglandin F receptor. Placenta. 2002;23(2-3):215-23.

[82] Smith SC, Baker PN, Symonds EM. Placental apoptosis in normal human pregnancy. Am J Obstet Gynecol. 1997;177:57-65.

[83] Larsen JF. Electron microscopy of the chorioallantoic placenta of the rabbit: II. The decidua and the maternal vessels. J Ultrastruct Res. 1963; 8(3-4): 327-338.

[84] Camm EJ, Hansell JA, Kane AD, Herrera EA, Lewis C, Wong S, et al. Partial contributions of developmental hypoxia and undernutrition to prenatal alterations in somatic growth and cardiovascular structure and function. Am J Obstet Gynecol. 2010;203(5):495.e24-34.

[85] Kwon H, Ford SP, Bazer FW, Spencer TE, Nathanielsz PW, Nijland MJ, et al. Maternal nutrient restriction reduces concentrations of amino acids and polyamines in ovine maternal and fetal plasma and fetal fluids. Biol Reprod. 2004;71(3):901-8.

[86] Sankaran S, Kyle PM. Aetiology and pathogenesis of IUGR. Best Pract Res Clin Obstet Gynaecol. 2009;23(6):765-77.

[87] Cohen E, Baerts W, van Bel F. Brain-Sparing in Intrauterine Growth Restriction: Considerations for the Neonatologist. Neonatology. 2015;108(4):269-76.

[88] Simitzis PE, Symeon GK, Kominakis AP, Bizelis IA, Chadio SE, Abas Z, et al. Severe maternal undernutrition and post-weaning behavior of rabbits. Physiol Behav. 2015;141:172-9.

[89] Chen LH, Liang L, Zhu WF, Wang YM, Zhu JF. Effects of intrauterine growth restriction and high-fat diet on serum lipid and transcriptional levels of related hepatic genes in rats. Zhongguo Dang Dai Er Ke Za Zhi. 2015;17(10):1124-30.

[90] George LA, Zhang L, Tuersunjiang N, Ma Y, Long NM, Uthlaut AB, et al. Early maternal undernutrition programs increased feed intake, altered glucose metabolism and insulin secretion,

and liver function in aged female offspring. Am J Physiol Regul Integr Comp Physiol. 2012;302(7):R795-804.

[91] Sookoian S, Gianotti TF, Burgueno AL, Pirola CJ. Fetal metabolic programming and epigenetic modifications: a systems biology approach. Pediatr Res. 2013;73(4 Pt 2):531-42.

[92] Gonzalez-Bulnes A, Astiz S, Vazquez-Gomez M, Garcia-Contreras C. Developmental origins of metabolic disorders: The need for biomarker candidates and therapeutic targets from adequate preclinical models. EuPA Open Proteomics. 2016.

[93] Ford SP, Hess BW, Schwope MM, Nijland MJ, Gilbert JS, Vonnahme KA, et al. Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. J Anim Sci. 2007;85(5):1285-94.

[94] Symeon GK, Goliomytis M, Bizelis I, Papadomichelakis G, Pagonopoulou O, Abas Z, et al. Effects of gestational maternal undernutrition on growth, carcass composition and meat quality of rabbit offspring. PLoS One. 2015;10(2):e0118259.

[95] Goliomytis M, Skoupa EP, Konga A, Symeon GK, Charismiadou MA, Deligeorgis SG. Influence of gestational maternal feed restriction on growth performance and meat quality of rabbit offsprings. Animal. 2016;10(1):157-62.

Chapter 4: Discussion

In human medicine, maternal undernutrition is a world-wide health issue, since nutritional imbalances during the pregnant stage may have significant consequences in the adequate development of the offspring. Among them, consequences range from miscarriage, IUGR, fetal programming and perinatal death (Triunfo & Lanzone, 2015) to life-threatening complications for expectant mothers (Vasiljevic *et al.,* 1996).

The same applies to other eutherian mammals. Thereby, many animal species have been used to unravel the pathogenesis of placental insufficiency and IUGR. Among them, rodents are the most recognized biomedical models and therefore the most frequently used for these studies (Schroder, 2003). However, nowadays, part of the society is concerned about the number and adequacy of animals used for scientific purposes. Hence, the employment of animals with a double functionality, biomedical modeling and livestock production, could reduce overlapping research and be a suitable alternative to the frequent employment of rodents. The rabbit is a well-established livestock animal in the Mediterranean area and, as previously exposed, this mammalian specie is the second most frequently used for scientific purposes in Europe (European Commision, 2013). However, the use of rabbits for pregnancy complications studies is scarce, despite hemodynamic changes and feto-placental features in this animal are much closer to humans (Derrick *et al.,* 2004; Polisca, *et al.,* 2010; Fischer *et al.,* 2012; Lecarpentier *et al.,* 2012) than what is in rodents.

Following this argument, the three works conducted within the period of this thesis were designed and performed with the double function that the rabbit possesses. In these studies, we employed the same level of MFR, 50% of the daily *ad libitum* non-pregnant state. The reason for employing a restriction based on preconception or *ad libitum* food intake, instead of maternal nutrient requirements for gestation, was that previous studies performed in other animal species had already reported that restriction based on *ad libitum* intake impairs placental development (Schulz *et al.,* 2012) and generates IUGR (Dywer *et al.,* 1995). Furthermore, by employing this approach, we ensured that the dietary treatment was customized to cover the necessities of each animal, avoiding possible over- or under-feeding that could alter our results. Moreover, the novelty of this thesis was that the restriction was applied in specific time windows of the rabbit gestation: during the preimplantation period (day 0-7 p.c.); from implantation to delivery (day 9-31 p.c.), and throughout gestation (day 0-31 p.c.). Combining these strategies, we have established a valuable rabbit model of IUGR by MFR.

Then, as we knew that currently there is non-effective treatment for IUGR available, we evaluated SC effectiveness for th e first time in a rabbit model of IUGR, as previously studied in the sheep model of undernutrition (Satterfield *et al.,* 2010) and in other animal models, such as knock outs (Stanley *et al.,* 2012b, 2015; Dilworth *et al.,* 2013), pharmacological treatments (Cauli *et al.,* 2010; Ramesar *et al.,* 2010; Herraiz *et al.,* 2012; Nassar *et al.,* 2012; Motta *et al.,* 2015) or other approaches (Miller *et al.,* 2009; George *et al.,* 2013; Luna *et al.,* 2015, 2016). Thereby, in this work, we were able to determine the consequences of MFR on pregnancy outcome, but at the same time, we characterized the effects of SC, which is currently being tested in pregnant women (Ganzevoort *et al.,* 2014).

In this thesis, we have employed different techniques to characterize placental function (*e.g.* Doppler ultrasonography or immunohistochemistry). Our results encompass the macroscopic and the microscopic features of IUGR. Thereby, this thesis provides an important view of the different processes and consequences that nutritional imbalances can generate in pregnant rabbits and in the develop of the offspring, with both points of view, the biomedical modeling and the livestock production. Moreover, taking into account the results obtained from these trials, we can establish what type of MFR strategy is the most suitable to induce IUGR, but we can also suggest whether the application of these feeding managements could be positive or not before the establishment in the industry.

In the first chapter, the global aim was to evaluate the suitability of the rabbit as a model and MFR as a valid approach to induce IUGR. The early diagnosis of IUGR was performed by echography and Doppler ultrasonography on day 21 of pregnancy (≈70% of the total pregnancy), being corroborated by *in vivo* measurements of the newborns. To achieve our goals, we restricted pregnant animals from day 9 of pregnancy, since, in this period, rabbit TBs begin to tap maternal blood vessels and gastrulation has already been concluded (Hoffman *et al.,* 1999). On the other hand, day 21 of pregnancy was selected as it is a period in which fetal viability is critical, since a significant change in the uteroplacental blood flow takes place, being more abundant the irrigation of the placenta than what is in the uterus (Hafez & Tsutsumi, 1996). Furthermore, by that time of gestation, organogenesis is assumed to be achieved (Beaudoin *et al.,* 2003). The main results of this first work evidenced that undernourished fetuses displayed reduction in their biometry, specifically a shorter occipito-nasal length respect well-nourished fetuses. Our results confirm the reliability of the occipito-nasal length as a valid measure to detect changes in fetal growth by

ultrasonography, as exposed by Kelly & Newnhamm (1989). Thus, undernourished fetuses denoted clear signs of hemodynamic alterations, as evidenced by lower end-diastolic velocities and significantly higher systolic peak and time-averaged mean velocities, which resulted in a trend for higher indexes of pulsatility and resistance at the UCA. These findings are important for characterizing the sequence of hemodynamic deterioration in the IUGR rabbit model and reinforce the UCA as an easy and reliable vessel to determine blood exchange (Acharya *et al.,* 2005). Furthermore, the low end-diastolic velocities observed in our trial are in line with the observation that in cases of placental insufficiency, end-diastolic velocities at the UCA decline (Baschat, 2011; Bansal *et al.,* 2016). Moreover, in this part of the study we observed a significant reduction in the cerebroplacental ratio. This ratio quantifies the redistribution of the cardiac output by dividing Doppler index from cerebral and fetoplacental vascularity and may suggest an early stage of the sequence of "brain sparing effect", although no direct changes at the MCA were observed. Our results support the use of this index and confirm that altered cerebroplacental ratio is indicative of adverse neonatal outcome, as observed in humans (DeVore, 2015; Ropacka-Lesiak *et al.,* 2015). The possible consequences of such redistribution in our model remain unclear. It has been proposed that brain sparing phenomenon is a "protective" effect to safeguard brain development and function (Torres-Rovira *et al.,* 2013). However, IUGR newborns with signs of brain sparing showed worse neurodevelopmental and behavioural outcomes than IUGR infants without signs of this mechanism (Cohen *et al.,* 2015). In rabbits, pups developed in an undernourished uterus showed altered eating, drinking and locomotor behaviors (Simitzis *et al.,* 2015), which ultimately could affect their growth performance in the farm. In our study, we corroborated that newborns developed in the harshest conditions were reduced in weight and size, clear signs of IUGR. Despite the restriction and the reduced phenotype of the offspring, mortality rate at birth was not increased. These results, along with the lack of significant changes in the weight of the mothers, reinforce the possible "conflict of interest" of the pregnant mother respect the growth of her offspring (Fowden & Moore, 2012). Thereby, the data obtained in the mortality rate demonstrates that the decision of the mother of not investing more resources to her litter was right despite its reduced morphometric outcome.

As soon as we validated our animal model of IUGR and our level of restriction, in the second chapter, we studied whether SC could allivate the effects of MFR on placenta development, fetal growth and offspring outcome. We also deepen our knowledge in the IUGR pathogenesis by the study of blood flow changes determined by Doppler ultrasonography, the possible changes in the

development of fetal organs and the potential metabolic alterations (carbohydrates and lipids) induced by MFR/IUGR and/or SC administration. We decided to start the treatment with SC (Viagra®) on day 22, as we knew from the first chapter that the incidence of IUGR is increased due to the high requirements of the fetus for growth. Previous studies in pregnant women observed promising results, improving maternal and fetal blood flow velocimetry and fetal wellbeing (Lacassie *et al.,* 2004; Lin *et al.,*2012; Panda *et al.,* 2014; Sun *et al.,* 2014). However, the study of the placenta was lacking.

The results of this trial were in line with what we previously observed in the first chapter. As we expected, MFR resulted in offspring suffering from IUGR on day 28 of pregnancy and at birth, but did not result in increased mortality rates of the offspring. MFR significantly reduced all morphometric parameters compared to well-nourished fetuses/newborns (crown-rump length, biparietal and thoracic diameters). Furthermore, at day 28 of pregnancy, these fetuses displayed significant alterations in the proportion of their organs, which are clear signs of asymmetric IUGR (Anderson, 1972). Undernourished fetuses showed reductions in head and trunk weights, which were associated to reduced weights of the brain and liver. Thus, undernourished fetuses evidenced increased brain and BLR ratios. The increase in such ratios is signs of blood flow distribution towards the brain (Anderson, 1972; Mitchell, 2001; Marton *et al.,* 2013). These findings support previous studies in which the raise of BLR was associated with undernutrition and dysmaturity conditions (Anderson 1972; Camm *et al.,* 2010). Moreover, when we performed the Doppler examination we observed altered hemodynamics in the MCA of these fetuses, specifically, increased systolic peak and time-averaged mean velocities, important findings that we did not observe in the first study. The raise in the systolic peak, which indirectly affected time-averaged mean velocity, has been reported in cases of fetal anemia (Mari, 2005), may be secondary to placental insufficiency.

Nevertheless, when we studied the metabolic profile of these fetuses, we observed that despite the MFR, undernourished fetuses did not develop hypoglycemia (common feature in IUGR; Hawdon *et al.,* 1993; Fafoula *et al.,* 2006). In fact, their normoglycemia was associated with similar plasma concentrations of cholesterol and triglycerides respect well-nourished fetuses. These findings may be explained by two different hypotheses. Firstly, glucose and lipid transporters in the placenta could be more efficient exchanging these products, maybe in response to hormonal signals driven by the undernourished fetuses (Gaccioli *et al.,* 2013). Other

option may involve that the placenta could adapt its metabolic rate favoring allocation of resources to the fetus over its own necessities. In this regard, previous experiments based on genetic and dietary treatments have demonstrated that the placenta can adapt its phenotype and function according to maternal and fetal interests (Fowden *et al.,* 2006; Sandovici *et al.,* 2012; Sferruzzi-Perri *et al.,* 2016). In fact, we found that MFR impaired placental structure phenotype (reduced length of the Db and Lz) and led to placental histopathological conditions (*e.g.* atrophy and fibrosis) without affecting placental weight mass. This last effect is important, as usually placenta weight positively correlates with growth parameters of the offspring (Soliman *et al.,* 2013). However, it has been demonstrated, in rats and sheep that an application of a 50% MFR results in heavier placentas (Heasman *et al.,* 1998; Langley-Evans *et al.,* 1996). This increment could be a compensatory mechanism of the placenta to alleviate nutritional deficits by expanding their surface area. Further stereological studies involving the determination of fetal capillary or maternal blood space volumes as well as diffusion capacity of the placenta are required. Finally, at the Doppler examination, we observed hemodynamic alterations in the placenta, specifically an increased systolic peak velocity at the UCA, as previously observed in the first study of this thesis and in mothers suffering from pregnancy complications (Landon *et al.,* 1989; Dicker *et al.,* 1990).

Regarding the effectiveness of SC, we confirmed that the administration of this drug could alleviate states of placental dysfunction; as these placentas showed significant changes when compared with those in the restricted group. Placentas treated with SC evidenced higher values of Lz ratio, which may be indicative of a compensation of the Lz to support fetal growth. Thus, absence of fibrosis and increased numbers of dilated small capillaries, venules and arterioles were found in these treated placentas. These features observed in the Lz are in line with previous works in mice. In this sense, Luna *et al.* 2015 found slightly vasodilation at the Lz. At the Db, SC increased the thickness and significantly altered arterial sinuses (hyperplasia and hypertrophy), which could have improved maternal blood flow towards the placenta, thereby facilitating maternal blood flow supply to the fetus. In this regard, the myometrium and the decidua vessels express high levels of PDE-5 (Buhimschi *et al.,* 2004; Coppage *et al.,* 2005) and, in the case of the rabbit, the placenta has a high expression of NOS, especially NOS3 (Khan *et al.,* 2012), which may facilitate SC function. Consequently, when we determined fetal growth, we observed that fetuses treated with SC significantly increased their size (higher crown-rump length and biparietal and thoracic diameters), but SC failed to improve fetal weight. Such findings were also observed at birth. The fact that SC could not improve fetal weight supports previous results

obtained in different rodent models (Ramesar *et al.,* 2010; George *et al.,* 2013; Motta *et al.,* 2015), but disagrees with the sheep model of undernutrition, in which fetal weight was increased by 14% (Satterfield *et al.,* 2010). These differences may be related to the length of the treatment (87 days *vs* 5 to 7-10 days), but also may be associated to differences in resource allocation between monotocous and polytocous species (Fowden & Moore, 2012).

Regarding fetal organs, SC could not revert the reductions in brain and liver weights associated to our model. However, SC significant increased the proportion of the liver respect the body weight of the fetus (liver ratio) compared to well- and under-nourished fetuses. Liver enlargement has been reported in rat fetuses exposed to SC (Pellicer *et al.,* 2011). Thus, fetuses treated with SC evidenced hyperglycemic states compared to the other two groups, which may be associated to the increased liver proportion. Surprisingly, when we studied the hemodynamic changes, we observed that this therapy increased the resistance indices (IP and IR) at the MCA, as observed by Dastjerdi *et al.* (2012) in pregnant women, which may suggest certain grade of vasoconstriction, as low indices reflect redistribution of cardiac output to the brain (Mari *et al.,* 2007). The fact that SC can cross the placenta (Pellicer *et al.,* 2011) and increase cGMP levels and cerebral blood flow (Zhang *et al.,* 2002; Li *et al.,* 2007) suggest us that the fetus may developed mechanisms to avoid excess of blood-flow supply in order to counteract possible adverse outcomes. Therefore, all these data should be taken with caution and further studies are required to corroborate the results in other time points of gestation as well as with other drug dosage. Moreover, elucidate whether the administration of this drug has postnatal consequences in the offspring health should be a main priority. Postnatal studies performed in mice exposed to SC in utero have observed that the offspring develops hypertension (increased systolic blood pressure) and reduced glucose sensitivity, suggesting metabolic alterations (Renshall, 2015). However, this last effect was only observed in female mice (Renshall, 2015). In contrast, SC can restore cognitive function but not motor activity in rats born from pre-eclamptic mothers (Cauli *et al.,* 2010). Future works should determine such effects in our rabbit model.

Finally, in the third chapter, we decided to explore other time windows of the rabbit gestation. We sought to determine the consequences of the 50% MFR during the preimplantational period (from day 0 to day 7 of the pregnancy) or throughout gestation (day 0 onwards). To have a global idea about the effects that MFR could generate in those periods of gestation, in this study, we evaluated all the "gestational compartments", mother, placenta and fetus. Specifically, we

assessed maternal metabolism throughout gestation by determining possible changes in leptin, plasma carbohydrates and lipid metabolites. Alterations in fetal and placental morphometric parameters were determined on day 28 of pregnancy. Thus, we also performed histopathological evaluation and apoptosis quantification in placental tissue in order to unravel possible mechanisms involved in the pathogenesis of placenta insufficiency and IUGR.

We observed that maternal body weight, plasma leptin concentrations and carbohydrates (glucose and fructosamine) were not affected by the MFR. Leptin is responsible for maintaining the metabolic state of the animal. This hormone can be considered as a good indicator of the metabolic status of the mother, as decreasing leptin concentrations may reflect a negative energetic balance (Menchetti *et al.,* 2015b). The lack of changes observed in leptin, along with the maintained glucose and fructosamine profiles observed in both restricted groups may suggest that, despite the restriction, food supplied was enough to cover maternal requirements. Our results are supported by previous works from Matsuoka *et al.* (2009), who found similar blood glucose levels in pregnant rabbits exposed to MFR, and disagree with the findings of Menchetti *et al.* (2015b). Lastly, the normoleptinemia found in these animals also disagree with the results exposed by Menchetti *et al.* (2015b). These steady levels of leptin suggest us that females could have had a positive energetic balance despite the restriction or may involve an increased production of leptin by placental and/or fetal compartments (Jelks *et al.,* 2009; Kirat *et al.,* 2015). In this regard, pregnant rats exposed to a 50% MFR showed a markedly increased in the circulating levels of leptin compared to well-nourished dams and this was associated to an upregulation of placental leptin (Jelks *et al.,* 2009).

In this study, we also observed that MFR was associated with alterations in maternal lipid profile, specifically in increments in plasma cholesterol and LDL-c concentrations and reduced levels of HDL-c and triglycerides. Most of the differences were observed between day 7 to day 14 of pregnancy, important periods for embryonic differentiation, implantation and early placentation (Weisbroth *et al.,* 1974; Fischer *et al.,* 2012). As previously exposed in the introduction, the first third of the pregnancy is usually considered as an anabolic phase due to the low requirements for growth of the developing embryo (Lain & Catalano, 2007). The altered lipid profiles found in undernourished mothers, in a more severe grade when the restriction was maintained throughout gestation, may suggest a physiological response of the dam to enhance implantation and placental formation. However, the increment of plasma cholesterol and LDL-c could have had a

rebound effect on embryo-fetal development and placental homeostasis, affecting gene expression at the blastocyst stage (Tarrade *et al.,* 2013) and may increasing free radicals by an excess of oxidative stress, which lastly could have disrupted the normal placentation (Chatzi *et al.,* 2009). Future research should be focus in these findings and its consequences in embryo patterning and early placentation.

In contrast, we have observed that plasma triglycerides were lower in the early stages of the pregnancy. This may suggest that the application of this level of MFR induced resource allocation strategies in the mothers to enhanced removal of triglycerides from circulation, increasing deposition of fat in maternal adipose tissue in the first third of gestation to support the exponential fetal growth in the last third of gestation and subsequent lactation (Chandi *et al.,* 2015). The results of this chapter show that undernourished mothers do not invest more than necessary in their offspring growth, which could be a good maternal strategy for future generations and for their own energetic balances (Fowden & Moore, 2012).

In line with previous studies of this thesis, we have observed that MFR disrupted fetal growth trajectory even when this feeding regimen was applied only during the preimplantation period. However, it was not associated to increased embryo-fetal mortality or altered implantation rates. Fetuses restricted during their whole period of gestation displayed significant reductions in size and weight. In contrast, those fetuses restricted only during the preimplantational period only evidenced reductions in their biometry (crown-rump length), as fetal weight was unchanged. These results support the idea that those mothers could have partially satisfied the requirements for growth of their offspring.

The assessment of the different fetal organs evidenced that both MFR strategies generate changes in the organogenesis. Specifically, liver weight was significantly reduced in both restricted groups respect to the *ad libitum* fed group. However, what was even more surprising was that the exposure to MFR only during preimplantational period resulted in altered proportions of the brain mass respect body weight. In this sense, animals exposed to such challenge evidenced altered brain ratios but no differences were found in total brain mass *versus* well-nourished fetuses, suggesting an enhanced and effective response of the fetus to prioritize brain development. However, BLR, indicative of asymmetric IUGR, was significantly higher in these animals; in line with the results observed in the fetuses developed in the harshest conditions

(throughout gestation). Future works should determine possible perinatal and postnatal brain abnormalities in these animals. Promising results may be obtained by magnetic resonance imaging (Simoes *et al.,* 2015) or metabolomics (Simoes *et al.,* 2016).

Finally, the study of the placentas revealed that the weight of this organ was unchanged in the preimplantational group. However, when MFR was carried out during the whole period of gestation, a significant reduction in the weight of the organ was found. Such finding is important to be remarked, as in the previous study (results 3.2) we did not observe changes in placental weight when we challenged pregnant rabbits from day 9 of gestation. This difference obtained using similar level of MFR, but applied in different time windows of gestation, may reinforce the preimplantational period and early stages of placental formation as critical points for placental establishment. Thus, it may suggest that the mother can compensate the early nutritional deficits from day 8 onwards. Future works should be focus in these early stages of placentation. However, histopathological findings in placental tissue have revealed that labyrinth stromal collapse is a common feature when MFR is applied, even if it is applied only during the preimplantation period. Finally, we have observed that apoptosis is a common biological phenomenon in placenta tissue and may be associated to placental remodeling (Smith *et al.,* 1997) and degenerative mechanism of the placenta near term (Mossman, 1926). However, what we have demonstrated is that imbalanced diets in gestation can increase apoptosis, which lastly may reduced nutrient and gases exchange, resulting in placenta insufficiency and IUGR. Thus, our study sets the bases on placental apoptosis in the rabbit placenta at term and is in line with the range of apoptosis rate reported in previous studies of MFR (Belkacemi *et al.,* 2009) and placental localization in mouse (Mu *et al.,* 2002), showing higher rates of apoptosis in the decidua with respect to the other zones of the placenta.

Overall, the data presented within this thesis suggest that MFR applied in different time windows of the rabbit gestation generate changes in maternal metabolism (associated to plasma lipids) without effect on maternal body weight. However, important changes in placental morphometry, function and homeostasis can occur even when MFR is applied only during preimplantational period, which may result in altered nutrient and oxygen transport to the fetus. Because of such impairments, fetal growth trajectory can be disrupted. However, these restrictions do not result in higher embryonic rate lost or higher intrauterine/early postnatal mortality rates of the offspring in any of the strategies employed. Nevertheless, the extent of the alterations observed in fetal and

neonatal morphometry will depend on the time of MFR application, being more severe when the MFR is carried out throughout gestation. These alterations are associated with the capacity of the mother to allocate her resources and the capability of the placenta to transfer them. Key organs such as brain and liver, which are critical for an adequate growth and health of the offspring can be altered. The insonation of the MCA has revealed that, in the rabbit model of undernutrition, the sequence of deterioration raises in the last third of gestation, as no direct effects in this vessel were observed on day 21 of gestation, but it did change on day 26 of gestation. This is in line with the idea that asymmetric IUGR fetuses are in a higher risk of suffering from brain damages as a consequence of the hypoxia and lack of nutrients. Consequently, the application of therapeutic interventions within this period of gestation could be beneficial to mitigate adverse cerebral and vascular outcomes. In this regard, the application of SC did not increase fetal or neonatal weight in our rabbit model of undernutrition. Thus, the administration of this drug, applied for the first time in a rabbit model, may imply alterations in brain networking formation, liver growth and hyperglycemic states in prenatal life that deserves further studies. However, SC counteracts reductions in perinatal body size and modifies placental growth and vascularization. Finally, taking into account all the data exposed in this thesis, the application of these feeding programs in rabbit farms should be done with caution. However, further research involving postnatal growth, meat characteristics and economic profitability of the farm are required.

Chapter 5a: Conclusions

Considering the experimental conditions of this research and based in the results obtained, the conclusions of this thesis are:

1) Maternal food restriction by 50% of the pre-gestational *ad libitum* intake during the rabbit gestation results in placental insufficiency and IUGR at birth, validating the rabbit as a suitable model of IUGR by maternal undernutrition. Fetal ecography and Doppler ultrasonography are adequate non-invasive methods to detect early reductions in the fetal biometry and fetoplacental blood flow alterations.

2) The protocols of maternal food restriction studied in this thesis do not affect maternal plasma leptin or glucidic metabolities, but alter maternal lipid metabolism during the periods of embryo development and early placental formation. Maternal food restriction induces histopathological changes in the placenta, which are mainly observed in the decidua and the labyrinth zone. Thereby, fetal growth trajectory is disrupted, impairing brain and liver development.

3) Sildenafil Citrate alleviates states of placental dysfunction and improves body size without increasing body weight in the rabbit offspring. Thereby, the administration of this drug could partially counteract the adverse effects of maternal food restriction. However, this therapy may imply hyperglycemic states in the fetus as well as possible blood overflow in the brain.

4) Maternal food restriction protocols should be applied with caution in livestock animals, since conceptus development can be disrupted and offspring growth at birth altered. These changes can affect postnatal growth and, therefore, modify the long-term productive goals. However, further studies addressing this issue are required.

Chapter 5b: Conclusiones

Considerando las condiciones experimentales de esta investigación y en base a los resultados obtenidos, las conclusiones de esta Tesis Doctoral son:

1) La aplicación de una restricción alimentaria a conejas gestantes, basada en una reducción del 50% sobre el consumo de pienso *ad libitum* en estado no gestante, genera insuficiencia placentaria y crecimiento intrauterino retardado a nacimiento. La ecografía fetal y la técnica Doppler son métodos no invasivos adecuados para la detección temprana de cambios en la biometría fetal y alteraciones en la hemodinámica fetoplacentaria.

2) Los protocolos de restricción alimentaria estudiados en la presente Tesis Doctoral no afectaron a las concentraciones plasmáticas de leptina ni el metabolismo glucídico materno. Sin embargo, sí modificaron al metabolismo lipídico en las primeras etapas embrionarias y de formación placentaria. La restricción alimentaria generó cambios histopatológicos en la placenta, principalmente en la zona de la decidua y del laberinto. Por consiguiente, el crecimiento fetal se vio alterado, así como el desarrollo del cerebro y del hígado.

3) El Citrato de Sildenafilo puede contrarrestar estados de insuficiencia placentaria inducidos por restricción alimentaria gestacional y mejorar el tamaño de la descendencia sin llegar a provocar un aumento del peso de la misma. Sin embargo, esta terapia puede implicar estados hiperglucémicos en el feto, así como un posible aumento del flujo sanguíneo a nivel cerebral.

4) Los protocolos de restricción alimentaria gestacional al 50% en hembras en producción se deben llevar a cabo con cierta cautela ya que inducen alteraciones en el desarrollo de la placenta, del feto y de los gazapos al nacimiento. Estos cambios podrían afectar el crecimiento postnatal y por tanto influir en los rendimientos productivos de la explotación a largo plazo, aunque más estudios son necesarios al respecto.

References

References

Acharya G, Wilsgaard T, Berntsen GK, Maltau JM, Kiserud T. Reference ranges for serial measurements of umbilical artery Doppler indices in the second half of pregnancy. Am J Obstet Gynecol. 2005;192(3):937-44.

Ahokas RA, Anderson GD, Lipshitz J. Effect of dietary restriction, during the last week only or throughout gestation, on cardiac output and uteroplacental blood flow in pregnant rats. J Nutr. 1983;113(1766):1776.

Akitake Y, Katsuragi S, Hosokawa M, Mishima K, Ikeda T, Miyazato M, et al. Moderate maternal food restriction in mice impairs physical growth, behavior, and neurodevelopment of offspring. Nutr Res. 2015;35(1):76-87.

Albu AR, Anca AF, Horhoianu VV, Horhoianu IA. Predictive factors for intrauterine growth restriction. J Med Life. 2014;7(2):165-71.

Alfirevic Z, Roberts D, Martlew V. How strong is the association between maternal thrombophilia and adverse pregnancy outcome? A systematic review. Eur J Obstet Gynecol Reprod Biol. 2002;101(1):6-14.

Alfirevic Z, Stampalija T, Gyte GM. Fetal and umbilical Doppler ultrasound in normal pregnancy. Cochrane Database Syst Rev. 2010(8):Cd001450.

Allain D, Renieri C. Genetics of fibre production and fleece characteristics in small ruminants, Angora rabbit and South American camelids. Animal. 2010;4(9):1472-81.

Allaire AD, Ballenger KA, Wells SR, McMahon MJ, Lessey BA. Placental apoptosis in preeclampsia. Obstet Gynecol. 2000;96(2):271-6.

Allison BJ, Brain KL, Niu Y, Kane AD, Herrera EA, Thakor AS, et al. Fetal in vivo continuous cardiovascular function during chronic hypoxia. J Physiol. 2016;594(5):1247-64.

Ananth CV, Smulian JC, Srinivas N, Getahun D, Salihu HM. Risk of infant mortality among twins in relation to placental abruption: contributions of preterm birth and restricted fetal growth. Twin Res Hum Genet. 2005;8(5):524-31.

Ananth CV, Vintzileos AM. Distinguishing pathological from constitutional small for gestational age births in population-based studies. Early Hum Dev. 2009;85(10):653-8.

Anderson JM. Increased brain weight-liver weight ratio as a necropsy sign of intrauterine undernutrition. J Clin Pathol. 1972;25(10):867-71.

Arias-Alvarez M. Influencia de diferentes estrategias reproductivas y nutricionales sobre la fisiologia ovarica en conejas primíparas [doctoral thesis]. [Madrid (Spain)]. Universidad Complutense de Madrid; 2011.

Arias-Alvarez M, Garcia-Garcia RM, Lopez-Tello J, Rebollar PG, Gutierrez-Adan A, Lorenzo PL. In vivo and in vitro maturation of rabbit oocytes differently affects the gene expression profile, mitochondrial distribution, apoptosis and early embryo development. Reprod Fertil Dev. 2016.

Ashworth CJ, Finch AM, Page KR, Nwagwu MO, McArdle HJ. Causes and consequences of fetal growth retardation in pigs. Reprod Suppl. 2001;58:233-46.

Ashworth CJ, Toma LM, Hunter MG. Nutritional effects on oocyte and embryo development in mammals: implications for reproductive efficiency and environmental sustainability. Philos Trans R Soc Lond B Biol Sci. 2009;364(1534):3351-61.

Baardman ME, Kerstjens-Frederikse WS, Berger RM, Bakker MK, Hofstra RM, Plosch T. The role of maternal-fetal cholesterol transport in early fetal life: current insights. Biol Reprod. 2013;88(1):24.

Bacon BJ, Gilbert RD, Kaufmann P, Smith AD, Trevino FT, Longo LD. Placental anatomy and diffusing capacity in guinea pigs following long-term maternal hypoxia. Placenta. 1984;5(6):475-87.

Baergen RN. Manual of Benirschke and Kaufmann's pathology of the human placenta. New York. Springer Science & Business Media. 2005.

Bakketeig LS. Current growth standards, definitions, diagnosis and classification of fetal growth retardation. Eur J Clin Nutr. 1998;52 Suppl 1:S1-4.

Bal'magiya TA, Surovtseva ZF. Growth pattern of rabbit fetuses during normal pregnancy and with inhibition of the “gestation dominant”. Bull Exp Biol Med. 1974;77(4):384-7.

Bansal S, Deka D, Dhadwal V, Mahendru R. Doppler changes as the earliest parameter in fetal surveillance to detect fetal compromise in intrauterine growth-restricted fetuses. Srp Arh Celok Lek. 2016;144(1-2):69-73.

Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia. 1993;36(1):62-7.

Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet. 1986;1(8489):1077-81.

Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. Lancet. 1989;2(8663):577-80.

Bartels A, Egan N, Broadhurst DI, Khashan AS, Joyce C, Stapleton M, et al. Maternal serum cholesterol levels are elevated from the 1st trimester of pregnancy: a cross-sectional study. J Obstet Gynaecol. 2012;32(8):747-52.

Baschat AA. Examination of the fetal cardiovascular system. Semin Fetal Neonatal Med. 2011;16(1):2-12.

Bassan H, Kidron D, Bassan M, Rotstein M, Kariv N, Giladi E, et al. The effects of vascular intrauterine growth retardation on cortical astrocytes. J Matern Fetal Neonatal Med. 2010;23(7):595-600.

Bassan H, Trejo LL, Kariv N, Bassan M, Berger E, Fattal A, et al. Experimental intrauterine growth retardation alters renal development. Pediatr Nephrol. 2000;15(3-4):192-5.

Batalle D, Munoz-Moreno E, Arbat-Plana A, Illa M, Figueras F, Eixarch E, et al. Long-term reorganization of structural brain networks in a rabbit model of intrauterine growth restriction. Neuroimage. 2014;100:24-38.

Battaglia FC, Meschia, G. An introduction to fetal physiology. Orlando, FL: Academic Press; 1986.

Bauer MK, Harding JE, Bassett NS, Breier BH, Oliver MH, Gallaher BH, et al. Fetal growth and placental function. Mol Cell Endocrinol. 1998;140(1-2):115-20.

Bauer R, Walter B, Brust P, Fuchtner F, Zwiener U. Impact of asymmetric intrauterine growth restriction on organ function in newborn piglets. Eur J Obstet Gynecol Reprod Biol. 2003;110 Suppl 1:S40-9.

Bauman DE, Currie WB. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J Dairy Sci. 1980;63(9):1514-29.

Baumann MU, Deborde S, Illsley NP. Placental glucose transfer and fetal growth. Endocrine. 2002;19(1):13-22.

Baumgartner C, Brandl J, Munch G, Ungerer M. Rabbit models to study atherosclerosis and its complications - Transgenic vascular protein expression in vivo. Prog Biophys Mol Biol. 2016;121(2):131-41.

Beaudoin S, Barbet P, Bargy F. Developmental stages in the rabbit embryo: guidelines to choose an appropriate experimental model. Fetal Diagn Ther. 2003;18(6):422-7.

Beck B, Ciszek M, Beyga Z. Animal Model for the Study of Intrauterine Growth Retardation. Fertil Steril. 2000; 74(3): S102.

Bekmukhambetov Y, Mamyrbayev A, Dzharkenov T, Kravtsova N, Utesheva Z, Tusupkaliev A, et al. Metabolic and immunologic aspects of fetoplacental insufficiency. Am J Reprod Immunol. 2016;76(4):299-306.

Belkacemi L, Chen CH, Ross MG, Desai M. Increased placental apoptosis in maternal food restricted gestations: role of the Fas pathway. Placenta. 2009;30(9):739-51.

Belkacemi L, Desai M, Nelson DM, Ross MG. Altered mitochondrial apoptotic pathway in placentas from undernourished rat gestations. Am J Physiol Regul Integr Comp Physiol. 2011b;301(6):R1599-615.

Belkacemi L, Jelks A, Chen CH, Ross MG, Desai M. Altered placental development in undernourished rats: role of maternal glucocorticoids. Reprod Biol Endocrinol. 2011a;9:105.

Bennis-Taleb N, Remacle C, Hoet JJ, Reusens B. A low-protein isocaloric diet during gestation affects brain development and alters permanently cerebral cortex blood vessels in rat offspring. J Nutr. 1999;129(8):1613-9.

Bergmann RL, Bergmann KE, Dudenhausen JW. Undernutrition and growth restriction in pregnancy. Nestle Nutr Workshop Ser Pediatr Program. 2008;61:103-21.

Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, et al. Maternal and child undernutrition: global and regional exposures and health consequences. Lancet. 2008;371(9608):243-60.

Blair EM, Nelson KB. Fetal growth restriction and risk of cerebral palsy in singletons born after at least 35 weeks' gestation. Am J Obstet Gynecol. 2015;212(4):520.e1-7.

Blankenship TN, Enders AC. Modification of uterine vasculature during pregnancy in macaques. Microsc Res Tech. 2003;60(4):390-401.

Blossner M, de Onis M (2005) Malnutrition: quantifying the health impact at national and local levels. Geneva: World Health Organization. 51 p.

Bor MV, Bor P, Cevik C. Serum fructosamine and fructosamine-albumin ratio as screening tests for gestational diabetes mellitus. Arch Gynecol Obstet. 1999;262(3-4):105-11.

Boyd EM. The effect of pregnancy and pseudopregnancy upon the blood lipoids of rabbits. J Physiol. 1936;86(3):250-7.

Bozzetti V, Tagliabue PE, Visser GH, van Bel F, Gazzolo D. Feeding issues in IUGR preterm infants. Early Hum Dev. 2013;89 Suppl 2:S21-3.

Brett KE, Ferraro ZM, Yockell-Lelievre J, Gruslin A, Adamo KB. Maternal-fetal nutrient transport in pregnancy pathologies: the role of the placenta. Int J Mol Sci. 2014;15(9):16153-85.

Buhimschi CS, Garfield RE, Weiner CP, Buhimschi IA. The presence and function of phosphodiesterase type 5 in the rat myometrium. Am J Obstet Gynecol. 2004;190(1):268-74.

Bujold E, Roberge S, Lacasse Y, Bureau M, Audibert F, Marcoux S, et al. Prevention of preeclampsia and intrauterine growth restriction with aspirin started in early pregnancy: a meta-analysis. Obstet Gynecol. 2010;116(2 Pt 1):402-14.

Burton GJ, Kaufmann P, Huppertz B. Anatomy and genesis of the Placenta. Amsterdam: Knobil and Nell's Physiology of Reproduction. 3rd ed. JD Neill ed. Academic Press; 2006.

Butte NF. Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. Am J Clin Nutr. 2000;71(5 Suppl):1256s-61s.

Calasans-Maia MD, Monteiro ML, Ascoli FO, Granjeiro JM. The rabbit as an animal model for experimental surgery. Acta Cir Bras. 2009;24(4):325-8.

Camm EJ, Hansell JA, Kane AD, Herrera EA, Lewis C, Wong S, et al. Partial contributions of developmental hypoxia and undernutrition to prenatal alterations in somatic growth and cardiovascular structure and function. Am J Obstet Gynecol. 2010;203(5):495.e24-34.

Campos PH, Silva BA, Donzele JL, Oliveira RF, Knol EF. Effects of sow nutrition during gestation on within-litter birth weight variation: a review. Animal. 2012;6(5):797-806.

Cappon GD, Fleeman TL, Chapin RE, Hurtt ME. Effects of feed restriction during organogenesis on embryo-fetal development in rabbit. Birth Defects Res B Dev Reprod Toxicol. 2005;74(5):424-30.

Carlin A, Alfirevic Z. Physiological changes of pregnancy and monitoring. Best Pract Res Clin Obstet Gynaecol. 2008;22(5):801-23.

Carney EW, Foote RH. Effects of superovulation, embryo recovery, culture system and embryo transfer on development of rabbit embryos in vivo and in vitro. J Reprod Fertil. 1990;89(2):543-51.

Carney EW, Scialli AR, Watson RE, DeSesso JM. Mechanisms regulating toxicant disposition to the embryo during early pregnancy: an interspecies comparison. Birth Defects Res C Embryo Today. 2004;72(4):345-60.

Carney EW, Tornesi B, Markham DA, Rasoulpour RJ, Moore N. Species-specificity of ethylene glycol-induced developmental toxicity: toxicokinetic and whole embryo culture studies in the rabbit. Birth Defects Res B Dev Reprod Toxicol. 2008;83(6):573-81.

Carter AM, Gothlin J, Olin T. An angiographic study of the structure and function of the uterine and maternal placental vasculature in the rabbit. J Reprod Fertil. 1971;25(2):201-10.

Carter RC, Jacobson JL, Sokol RJ, Avison MJ, Jacobson SW. Fetal alcohol-related growth restriction from birth through young adulthood and moderating effects of maternal prepregnancy weight. Alcohol Clin Exp Res. 2013;37(3):452-62.

Catalano PM, Tyzbir ED, Wolfe RR, Roman NM, Amini SB, Sims EA. Longitudinal changes in basal hepatic glucose production and suppression during insulin infusion in normal pregnant women. Am J Obstet Gynecol. 1992;167(4 Pt 1):913-9.

Cauli O, Herraiz S, Pellicer B, Pellicer A, Felipo V. Treatment with sildenafil prevents impairment of learning in rats born to pre-eclamptic mothers. Neuroscience. 2010;171(2):506-12.

Chandi A, Sirohiwal D, Malik R. Association of early maternal hypertriglyceridemia with pregnancy-induced hypertension. Arch Gynecol Obstet. 2015;292(5):1135-43.

Chang JH, Rutledge JC, Stoops D, Abbe R. Hypobaric hypoxia-induced intrauterine growth retardation. Biol Neonate. 1984;46(1):10-3.

Chatzi L, Plana E, Daraki V, Karakosta P, Alegkakis D, Tsatsanis C, et al. Metabolic syndrome in early pregnancy and risk of preterm birth. Am J Epidemiol. 2009;170(7):829-36.

Chavatte-Palmer P, Guillomot M. Comparative implantation and placentation. Gynecol Obstet Invest. 2007;64(3):166-74.

Chavatte-Palmer P, Laigre P, Simonoff E, Chesne P, Challah-Jacques M, Renard JP. In utero characterisation of fetal growth by ultrasound scanning in the rabbit. Theriogenology. 2008;69(7):859-69.

Chavatte-Palmer P, Tarrade A, Kiefer H, Duranthon V, Jammes H. Breeding animals for quality products: not only genetics. Reprod Fertil Dev. 2016;28(1-2):94-111.

Chavatte-Palmer P, Richard C, Peugnet P, Robles M, Rousseau-Ralliard D, Tarrade A. The developmental origins of health and disease: importance for animal production. Anim Reprod. 2015;12:505-20.

Chen PY, Ganguly A, Rubbi L, Orozco LD, Morselli M, Ashraf D, et al. Intrauterine calorie restriction affects placental DNA methylation and gene expression. Physiol Genomics. 2013;45(14):565-76.

Cnossen JS, Morris RK, ter Riet G, Mol BW, van der Post JA, Coomarasamy A, et al. Use of uterine artery Doppler ultrasonography to predict pre-eclampsia and intrauterine growth restriction: a systematic review and bivariable meta-analysis. Cmaj. 2008;178(6):701-11.

Coan PM, Fowden AL, Constancia M, Ferguson-Smith AC, Burton GJ, Sibley CP. Disproportional effects of Igf2 knockout on placental morphology and diffusional exchange characteristics in the mouse. J Physiol. 2008;586(20):5023-32.

Coan PM, Vaughan OR, Sekita Y, Finn SL, Burton GJ, Constancia M, et al. Adaptations in placental phenotype support fetal growth during undernutrition of pregnant mice. J Physiol. 2010;588(Pt 3):527-38.

Cohen E, Baerts W, van Bel F. Brain-Sparing in Intrauterine Growth Restriction: Considerations for the Neonatologist. Neonatology. 2015;108(4):269-76.

Coppage KH, Sun X, Baker RS, Clark KE. Expression of phosphodiesterase 5 in maternal and fetal sheep. Am J Obstet Gynecol. 2005;193(3 Pt 2):1005-10.

Cordier AG, Leveille P, Dupont C, Tarrade A, Picone O, Larcher T, et al. Dietary lipid and cholesterol induce ovarian dysfunction and abnormal LH response to stimulation in rabbits. PLoS One. 2013;8(5):e63101.

Dalle Zotte A. Rabbit farming for meat purposes. Animal Frontiers. 2014;4(4):62-67.

Darinskii NV, Surovtseva ZF. Effect of the conditions of antenatal development on functional maturation of rabbit fetal skeletal muscle. Bull Exp Biol Med. 1974;77(2):104-6.

Dastjerdi MV, Hosseini S, Bayani L. Sildenafil citrate and uteroplacental perfusion in fetal growth restriction. J Res Med Sci. 2012;17(7):632-6.

Davison BB, Cogswell FB, Baskin GB, Falkenstein KP, Henson EW, Krogstad DJ. Placental changes associated with fetal outcome in the Plasmodium coatneyi/rhesus monkey model of malaria in pregnancy. Am J Trop Med Hyg. 2000;63(3-4):158-73.

De Blas C, Wiseman J. Nutrition of the rabbit. London: CABI Publishing; 2003.

de Rooij SR, Wouters H, Yonker JE, Painter RC, Roseboom TJ. Prenatal undernutrition and cognitive function in late adulthood. Proc Natl Acad Sci U S A. 2010;107(39):16881-6.

De Santis M, Cesari E, Nobili E, Straface G, Cavaliere AF, Caruso A. Radiation effects on development. Birth Defects Res C Embryo Today. 2007;81(3):177-82.

Denker HW, Gerdes HJ. The dynamic structure of rabbit blastocyst coverings. I. Transformation during regular preimplantation development. Anat Embryol (Berl). 1979;157(1):15-34.

Derrick M, Drobyshevsky A, Ji X, Tan S. A model of cerebral palsy from fetal hypoxia-ischemia. Stroke. 2007;38(2 Suppl):731-5.

Derrick M, Luo NL, Bregman JC, Jilling T, Ji X, Fisher K, et al. Preterm fetal hypoxia-ischemia causes hypertonia and motor deficits in the neonatal rabbit: a model for human cerebral palsy? J Neurosci. 2004;24(1):24-34.

DeSesso JM. Comparative embryology. In: Hood R, editor. Handbook of developmental toxicology. CRC Press; 1997. pp. 111–174.

DeVore GR. The importance of the cerebroplacental ratio in the evaluation of fetal well-being in SGA and AGA fetuses. Am J Obstet Gynecol. 2015;213(1):5-15.

Diaz P, Powell TL, Jansson T. The role of placental nutrient sensing in maternal-fetal resource allocation. Biol Reprod. 2014;91(4):82.

Dicker D, Goldman JA, Yeshaya A, Peleg D. Umbilical artery velocimetry in insulin dependent diabetes mellitus (IDDM) pregnancies. J Perinat Med. 1990;18(5):391-5.

DiFederico E, Genbacev O, Fisher SJ. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. Am J Pathol. 1999;155(1):293-301.

Dilworth MR, Kusinski LC, Cowley E, Ward BS, Husain SM, Constancia M, et al. Placental-specific Igf2 knockout mice exhibit hypocalcemia and adaptive changes in placental calcium transport. Proc Natl Acad Sci U S A. 2010;107(8):3894-9.

Dilworth MR, Andersson I, Renshall LJ, Cowley E, Baker P, Greenwood S, et al. Sildenafil citrate increases fetal weight in a mouse model of fetal growth restriction with a normal vascular phenotype. PLoS One. 2013;8(10):e77748.

Douglas AJ, Johnstone LE, Leng G. Neuroendocrine mechanisms of change in food intake during pregnancy: a potential role for brain oxytocin. Physiol Behav. 2007;91(4):352-65.

Drobyshevsky A, Derrick M, Wyrwicz AM, Ji X, Englof I, Ullman LM, et al. White matter injury correlates with hypertonia in an animal model of cerebral palsy. J Cereb Blood Flow Metab. 2007;27(2):270-81.

Duff GL, Brechin DJ, Finkelstein WE. The effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. IV. The effect of insulin therapy on the inhibition of atherosclerosis in the alloxan-diabetic rabbit. J Exp Med. 1954;100(4):371-80.

Duff GL, Mc MG. The effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. J Exp Med. 1949;89(6):611-30.

Duff GL, Payne TP. The effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. III. The mechanism of the inhibition of experimental cholesterol atherosclerosis in alloxan-diabetic rabbits. J Exp Med. 1950;92(4):299-317.

Dwyer CM, Madgwick AJ, Ward SS, Stickland NC. Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea-pig. Reprod Fertil Dev. 1995;7(5):1285-92.

Eixarch E, Batalle D, Illa M, Munoz-Moreno E, Arbat-Plana A, Amat-Roldan I, et al. Neonatal neurobehavior and diffusion MRI changes in brain reorganization due to intrauterine growth restriction in a rabbit model. PLoS One. 2012;7(2):e31497.

Eixarch E, Figueras F, Hernandez-Andrade E, Crispi F, Nadal A, Torre I, et al. An experimental model of fetal growth restriction based on selective ligature of uteroplacental vessels in the pregnant rabbit. Fetal Diagn Ther. 2009;26(4):203-11.

Eixarch E, Hernandez-Andrade E, Crispi F, Illa M, Torre I, Figueras F, et al. Impact on fetal mortality and cardiovascular Doppler of selective ligature of uteroplacental vessels compared with undernutrition in a rabbit model of intrauterine growth restriction. Placenta. 2011;32(4):304-9.

Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516.

European Commision. Seventh Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States. Brussels. 2013:40 p.

Fafoula O, Alkhayyat H, Hussain K. Prolonged hyperinsulinaemic hypoglycaemia in newborns with intrauterine growth retardation. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2006; 91(6), F467-F467.

Fan J, Kitajima S, Watanabe T, Xu J, Zhang J, Liu E, et al. Rabbit models for the study of human atherosclerosis: from pathophysiological mechanisms to translational medicine. Pharmacol Ther. 2015;146:104-19.

Food and Agriculture Organization of the United Nations (FAO). The state of food insecurity in the world. Rome: FAO. 2015: 56 p.

Fazleabas AT. Physiology and pathology of implantation in the human and nonhuman primate. Semin Reprod Med. 2007;25(6):405-9.

Feist H, Blocker T, Hussein K. Massive perivillous fibrin deposition, chronic histiocytic intervillositis and villitis of unknown etiology: Lesions of the placenta at the fetomaternal interface with risk of recurrence. Pathologe. 2015;36(4):355-61.

Feld J, Kibari A, Rozenbaum M, Riskin-Mashiah S, Eder L, Laor A, et al. The fetal outcomes of pregnancies of systemic lupus erythematosus patients in northern Israel. J Matern Fetal Neonatal Med. 2015;28(5):564-7.

Ferrer-Vaquer A, Hadjantonakis AK. Birth defects associated with perturbations in preimplantation, gastrulation, and axis extension: from conjoined twinning to caudal dysgenesis. Wiley Interdiscip Rev Dev Biol. 2013;2(4):427-42.

Figueras F, Gardosi J. Intrauterine growth restriction: new concepts in antenatal surveillance, diagnosis, and management. Am J Obstet Gynecol. 2011;204(4):288-300.

Figueroa H, Cifuentes J, Lozano M, Alvarado C, Cabezas C, Eixarch E, et al. Nitric oxide synthase and changes in oxidative stress levels in embryonic kidney observed in a rabbit model of intrauterine growth restriction. Prenat Diagn. 2016;36(7):628-35

Figueroa H, Lozano M, Suazo C, Eixarch E, Illanes SE, Carreno JE, et al. Intrauterine growth restriction modifies the normal gene expression in kidney from rabbit fetuses. Early Hum Dev. 2012;88(11):899-904.

Fischer B, Chavatte-Palmer P, Viebahn C, Navarrete Santos A, Duranthon V. Rabbit as a reproductive model for human health. Reproduction. 2012;144(1):1-10.

Fleming TP, Lucas ES, Watkins AJ, Eckert JJ. Adaptive responses of the embryo to maternal diet and consequences for post-implantation development. Reprod Fertil Dev. 2011;24(1):35-44.

Foote RH, Carney EW. The rabbit as a model for reproductive and developmental toxicity studies. Reprod Toxicol. 2000;14(6):477-93.

Fortun-Lamothe L. Energy balance and reproductive performance in rabbit does. Anim Reprod Sci. 2006;93(1-2):1-15.

Fortun-Lamothe L. Effects of dietary fat on reproductive performance of rabbit does: a review. World Rabbit Sci. 2010;5(1):33-38.

Fortun-Lamothe L, Lebas F. Effects of dietary energy level and source on foetal development and energy balance in concurrently pregnant and lactating primiparous rabbit does. Anim Sci. 1996;62(03):615-620.

Fowden AL, Moore T. Maternal-fetal resource allocation: co-operation and conflict. Placenta. 2012;33 Suppl 2:e11-5.

Fowden AL, Sferruzzi-Perri AN, Coan PM, Constancia M, Burton GJ. Placental efficiency and adaptation: endocrine regulation. J Physiol. 2009;587(Pt 14):3459-72.

Fowden AL, Ward JW, Wooding FP, Forhead AJ, Constancia M. Programming placental nutrient transport capacity. J Physiol. 2006;572(Pt 1):5-15.

Frantz E, Menezes HS, Lange KC, Abegg MP, Correa CA, Zangalli L, et al. The effect of maternal hypercholesterolemia on the placenta and fetal arteries in rabbits. Acta Cir Bras. 2012;27(1):7-12.

Franzek EJ, Sprangers N, Janssens AC, Van Duijn CM, Van De Wetering BJ. Prenatal exposure to the 1944-45 Dutch 'hunger winter' and addiction later in life. Addiction. 2008;103(3):433-8.

Freemark M. Regulation of maternal metabolism by pituitary and placental hormones: roles in fetal development and metabolic programming. Horm Res. 2006;65 Suppl 3:41-9.

Froen JF, Gardosi JO, Thurmann A, Francis A, Stray-Pedersen B. Restricted fetal growth in sudden intrauterine unexplained death. Acta Obstet Gynecol Scand. 2004;83(9):801-7.

Fu J, Olofsson P. Fetal ductus venosus, middle cerebral artery and umbilical artery flow responses to uterine contractions in growth-restricted human pregnancies. Ultrasound Obstet Gynecol. 2007;30(6):867-73.

Furukawa S, Kuroda Y, Sugiyama A. A comparison of the histological structure of the placenta in experimental animals. J Toxicol Pathol. 2014;27(1):11-8.

Gaccioli F, Lager S, Powell TL, Jansson T. Placental transport in response to altered maternal nutrition. J Dev Orig Health Dis. 2013;4(2):101-15.

Ganguly A, Chen Y, Shin BC, Devaskar SU. Prenatal caloric restriction enhances DNA methylation and MeCP2 recruitment with reduced murine placental glucose transporter isoform 3 expression. J Nutr Biochem. 2014;25(2):259-66.

Ganguly A, Collis L, Devaskar SU. Placental glucose and amino acid transport in calorie-restricted wild-type and Glut3 null heterozygous mice. Endocrinology. 2012;153(8):3995-4007.

Ganzevoort W, Alfirevic Z, von Dadelszen P, Kenny L, Papageorghiou A, van Wassenaer-Leemhuis A, et al. STRIDER: Sildenafil Therapy In Dismal prognosis Early-onset intrauterine growth Restriction--a protocol for a systematic review with individual participant data and aggregate data meta-analysis and trial sequential analysis. Syst Rev. 2014;3:23.

Gao F, Hou XZ, Liu YC, Wu SQ, Ao CJ. Effect of maternal under-nutrition during late pregnancy on lamb birth weight. Asian Australas J Anim Sci. 2008;21(3):371.

Gao F, Liu YC, Hou XZ. Effect of maternal undernutrition during late pregnancy on growth and development of ovine fetal visceral organs. Asian Australas J Anim Sci. 2009;22(12):1633-1639.

Genbacev O, DiFederico E, McMaster M, Fisher SJ. Invasive cytotrophoblast apoptosis in pre-eclampsia. Hum Reprod. 1999;14 Suppl 2:59-66.

George EM, Palei AC, Dent EA, Granger JP. Sildenafil attenuates placental ischemia-induced hypertension. Am J Physiol Regul Integr Comp Physiol. 2013;305(4):R397-403.

Georgiades P, Ferguson-Smith AC, Burton GJ. Comparative developmental anatomy of the murine and human definitive placentae. Placenta. 2002;23(1):3-19.

Getz GS, Reardon CA. Animal models of atherosclerosis. Arterioscler Thromb Vasc Biol. 2012;32(5):1104-15.

Gilbert M, Jost A. Hyperglycemia and glycogen storage in the rabbit fetal liver. Role of age and hormonal status. Biol Neonate. 1977;32(3-4):125-31.

Gilbert RD, Cummings LA, Juchau MR, Longo LD. Placental diffusing capacity and fetal development in exercising or hypoxic guinea pigs. J Appl Physiol Respir Environ Exerc Physiol. 1979;46(4):828-34.

Giussani DA. The fetal brain sparing response to hypoxia: physiological mechanisms. J Physiol. 2016;594(5):1215-30.

Giussani DA. The vulnerable developing brain. Proc Natl Acad Sci U S A. 2011;108(7):2641-2.

Godfrey KM, Haugen G, Kiserud T, Inskip HM, Cooper C, Harvey NC, et al. Fetal liver blood flow distribution: role in human developmental strategy to prioritize fat deposition versus brain development. PLoS One. 2012;7(8):e41759.

Goldberg G. Nutrition in pregnancy and lactation. In: Shetty P, editor. Nutrition Through the Life Cycle. Leatherhead Publishing; 2002. pp. 63-90.

Goldin E, Harel S, Tomer A, Yavin E. Arachidonic acid oxidation by brain and placenta preparations from normal and placental insufficient fetal rabbit. J Neurochem. 1987;48(3):695-701.

Goliomytis M, Skoupa EP, Konga A, Symeon GK, Charismiadou MA, Deligeorgis SG. Influence of gestational maternal feed restriction on growth performance and meat quality of rabbit offsprings. Animal. 2016;10(1):157-62.

Gonzalez PN, Gasperowicz M, Barbeito-Andres J, Klenin N, Cross JC, Hallgrimsson B. Chronic Protein Restriction in Mice Impacts Placental Function and Maternal Body Weight before Fetal Growth. PLoS One. 2016;11(3):e0152227.

Gonzalez-Bulnes A, Astiz S, Ovilo C, Lopez-Bote CJ, Torres-Rovira L, Barbero A, et al. Developmental Origins of Health and Disease in swine: implications for animal production and biomedical research. Theriogenology. 2016b;86(1):110-9.

Gonzalez-Bulnes A, Astiz S, Parraguez VH, Garcia-Contreras C, Vazquez-Gomez M. Empowering Translational Research in Fetal Growth Restriction: Sheep and Swine Animal Models. Curr Pharm Biotechnol. 2016a;17(10):848-55.

Gonzalez-Bulnes A, Ovilo C. Genetic basis, nutritional challenges and adaptive responses in the prenatal origin of obesity and type-2 diabetes. Curr Diabetes Rev. 2012;8(2):144-54.

Gonzalez-Bulnes A, Torres-Rovira L, Astiz S, Ovilo C, Sanchez-Sanchez R, Gomez-Fidalgo E, et al. Fetal Sex Modulates Developmental Response to Maternal Malnutrition. PLoS One. 2015;10(11):e0142158.

Gonzalez-Tendero A, Torre I, Garcia-Canadilla P, Crispi F, Garcia-Garcia F, Dopazo J, et al. Intrauterine growth restriction is associated with cardiac ultrastructural and gene expression changes related to the energetic metabolism in a rabbit model. Am J Physiol Heart Circ Physiol. 2013;305(12):H1752-60.

Graur D, Duret L, Gouy M. Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). Nature. 1996;379(6563):333-5.

Grivell R, Dodd J, Robinson J. The prevention and treatment of intrauterine growth restriction. Best Pract Res Clin Obstet Gynaecol. 2009;23(6):795-807.

Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res. 2004;114(5-6):397-407.

Gui K, Ren W, Yu Y, Li X, Dong J, Yin W. Inhibitory effects of platelet-rich plasma on intervertebral disc degeneration: a preclinical study in a rabbit model. Med Sci Monit. 2015;21:1368-75.

Gutaj P, Wender-Ozegowska E, Iciek R, Zawiejska A, Pietryga M, Brazert J. Maternal serum placental growth factor and fetal SGA in pregnancy complicated by type 1 diabetes mellitus. J Perinat Med. 2014;42(5):629-33.

Haas JD, Moreno-Black G, Frongillo EA, Jr., Pabon J, Pareja G, Ybarnegaray J, et al. Altitude and infant growth in Bolivia: a longitudinal study. Am J Phys Anthropol. 1982;59(3):251-62.

Hadden DR, McLaughlin C. Normal and abnormal maternal metabolism during pregnancy. Semin Fetal Neonatal Med. 2009;14(2):66-71.

Hafez ES, Tsutsumi Y. Changes in endometrial vascularity during implantation and pregnancy in the rabbit. Am J Anat. 1966;118(1):249-82.

Hales CN, Barker DJ. The thrifty phenotype hypothesis. Br Med Bull. 2001;60:5-20.

Han Z, Lutsiv O, Mulla S, McDonald SD. Maternal height and the risk of preterm birth and low birth weight: a systematic review and meta-analyses. J Obstet Gynaecol Can. 2012;34(8):721-46.

Haneda R, Mizoguchi Y, Matsuoka T, Mizuguchi H, Endoh T, Fukuda K, et al. Changes in blood parameters in pregnant Japanese White rabbits. J Toxicol Sci. 35. Japan2010. p. 773-8.

Haque SF, Izumi S, Aikawa H, Suzuki T, Matsubayashi H, Murano T, et al. Anesthesia and acoustic stress-induced intra-uterine growth retardation in mice. J Reprod Dev. 2004;50(2):185-90.

Hardy K. Apoptosis in the human embryo. Rev Reprod. 1999;4(3):125-34.

Harel S, Watanabe K, Linke I, Schain RJ. Growth and development of the rabbit brain. Biol Neonate. 1972;21(5):381-99.

Harel S, Yavin E, Tomer A, Barak Y, Binderman I. Brain: body ratio and conceptional age in vascular-induced intrauterine growth retarded rabbits. Brain Dev. 1985;7(6):575-9.

Harper JL, Caesar GA, Pennington KA, Davis JW, Schulz LC. Placental changes caused by food restriction during early pregnancy in mice are reversible. Reproduction. 2015;150(3):165-72.

Hasegawa J, Matsuoka R, Ichizuka K, Sekizawa A, Farina A, Okai T. Velamentous cord insertion into the lower third of the uterus is associated with intrapartum fetal heart rate abnormalities. Ultrasound Obstet Gynecol. 2006;27(4):425-9.

Haucke E, Navarrete Santos A, Simm A, Henning C, Glomb MA, Gurke J, et al. Accumulation of advanced glycation end products in the rabbit blastocyst under maternal diabetes. Reproduction. 2014;148(2):169-78.

Haugel S, Leturque A, Gilbert M, Kande J, Girard J. Glucose utilization by the placenta and fetal tissues in fed and fasted pregnant rabbits. Pediatr Res. 1988;23(5):480-3.

Hauguel-de Mouzon S, Lepercq J. Placental leptin and pregnancy pathologies. Gynecol Obstet Fertil. 2001;29(7-8):534-7.

Hawdon JM, Weddell A, Aynsley-Green A, Ward Platt MP. Hormonal and metabolic response to hypoglycaemia in small for gestational age infants. Arch Dis Child. 1993;68(3 Spec No):269-73.

Hay WW, Jr. Recent observations on the regulation of fetal metabolism by glucose. J Physiol. 2006;572(Pt 1):17-24.

Hayashi K, Kusakabe KT, Khan H, Kuniyoshi N, Takeshita A, Hiyama M, et al. Characteristic patterns of maternal and fetal arterial construction in the rabbit placenta. Med Mol Morphol. 2014;47(2):76-82.

Heasman L, Clarke L, Firth K, Stephenson T, Symonds ME. Influence of restricted maternal nutrition in early to mid gestation on placental and fetal development at term in sheep. Pediatr Res. 1998;44(4):546-51.

Heasman L, Clarke L, Stephenson TJ, Symonds ME. The influence of maternal nutrient restriction in early to mid-pregnancy on placental and fetal development in sheep. Proc Nutr Soc. 1999;58(2):283-8.

Herraiz S, Pellicer B, Serra V, Cauli O, Cortijo J, Felipo V, et al. Sildenafil citrate improves perinatal outcome in fetuses from pre-eclamptic rats. Bjog. 2012;119(11):1394-402.

Herrera E, Ortega-Senovilla H. Disturbances in lipid metabolism in diabetic pregnancy - Are these the cause of the problem? Best Pract Res Clin Endocrinol Metab. 2010;24(4):515-25.

Herrera E. Lipid metabolism in pregnancy and its consequences in the fetus and newborn. Endocrine. 2002;19(1):43-55.

Herrera EA, Pulgar VM, Riquelme RA, Sanhueza EM, Reyes RV, Ebensperger G, et al. High-altitude chronic hypoxia during gestation and after birth modifies cardiovascular responses in newborn sheep. Am J Physiol Regul Integr Comp Physiol. 2007;292(6):R2234-40.

Herrera EA, Rojas RT, Krause BJ, Ebensperger G, Reyes RV, Giussani DA, et al. Cardiovascular function in term fetal sheep conceived, gestated and studied in the hypobaric hypoxia of the Andean altiplano. J Physiol. 2016;594(5):1231-45.

Herrera E., Lasuncion MA. Maternal-fetal transfer of lipid metabolites. In: Polin RA, Fox WW, Abman SH, editors. Fetal and Neonatal Physiology. Elsevier; 1998. pp. 375-388.

Higgins JS, Vaughan OR, Fernandez de Liger E, Fowden AL, Sferruzzi-Perri AN. Placental phenotype and resource allocation to fetal growth are modified by the timing and degree of hypoxia during mouse pregnancy. J Physiol. 2015;594(5):1341-56.

Hoffman LH, Breinan DR, Blaeuer GL. The rabbit as a model for implantation: in vivo and in vitro studies. In: Carson DD, editor. Embryo Implantation. Springer; 1999. pp. 151-160.

Hofhuis W, de Jongste JC, Merkus PJ. Adverse health effects of prenatal and postnatal tobacco smoke exposure on children. Arch Dis Child. 2003;88(12):1086-90.

Houlton MC, Marivate M, Philpott RH. The prediction of fetal growth retardation in twin pregnancy. Br J Obstet Gynaecol. 1981;88(3):264-73.

Huang C, Phillips MR, Zhang Y, Zhang J, Shi Q, Song Z, et al. Malnutrition in early life and adult mental health: evidence from a natural experiment. Soc Sci Med. 2013;97:259-66.

Hue-Beauvais C, Chavatte-Palmer P, Aujean E, Dahirel M, Laigre P, Pechoux C, et al. An obesogenic diet started before puberty leads to abnormal mammary gland development during pregnancy in the rabbit. Dev Dyn. 2011;240(2):347-56.

Hue-Beauvais C, Koch E, Chavatte-Palmer P, Galio L, Chat S, Letheule M, et al. Milk from dams fed an obesogenic diet combined with a high-fat/high-sugar diet induces long-term abnormal mammary gland development in the rabbit. J Anim Sci. 2015;93(4):1641-55.

Hui JY, Hoffmann M, Kumar G. Embryo-fetal exposure and developmental outcome of thalidomide following oral and intravaginal administration to pregnant rabbits. Reprod Toxicol. 2014;48:115-23.

Hult M, Tornhammar P, Ueda P, Chima C, Bonamy AK, Ozumba B, et al. Hypertension, diabetes and overweight: looming legacies of the Biafran famine. PLoS One. 2010;5(10):e13582.

Huppertz B, Kadyrov M, Kingdom JC. Apoptosis and its role in the trophoblast. Am J Obstet Gynecol. 2006;195(1):29-39.

Illa M, Eixarch E, Batalle D, Arbat-Plana A, Munoz-Moreno E, Figueras F, et al. Long-term functional outcomes and correlation with regional brain connectivity by MRI diffusion tractography metrics in a near-term rabbit model of intrauterine growth restriction. PLoS One. 2013;8(10):e76453.

Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 2001;15(23):3059-87.

Jaattela M. Programmed cell death: many ways for cells to die decently. Ann Med. 2002;34(6):480-8.

Javadi S, Asri-Rezaei S, Allahverdizadeh M. Interrelationship of betaeta-2 microglobulin, blood urea nitrogen and creatinine in streptozotocin-induced diabetes mellitus in rabbits. Vet Res Forum. 2014;5(1):7-11.

Jelks A, Belkacemi L, Han G, Chong WL, Ross MG, Desai M. Paradoxical increase in maternal plasma leptin levels in food-restricted gestation: contribution by placental and adipose tissue. Reprod Sci. 2009;16(7):665-75.

Jensen A, Garnier Y, Berger R. Dynamics of fetal circulatory responses to hypoxia and asphyxia. Eur J Obstet Gynecol Reprod Biol. 1999;84(2):155-72.

Kalhan S, Parimi P. Gluconeogenesis in the fetus and neonate. Semin Perinatol. 2000;24(2):94-106.

Kampman MA, Bilardo CM, Mulder BJ, Aarnoudse JG, Ris-Stalpers C, van Veldhuisen DJ, et al. Maternal cardiac function, uteroplacental Doppler flow parameters and pregnancy outcome: a systematic review. Ultrasound Obstet Gynecol. 2015;46(1):21-8.

Kane MT. Inhibition of zona shedding of rabbit blastocysts in culture by the presence of a mucin coat. J Reprod Fertil. 1975;44(3):539-42.

Kelly RW, Newnham JP. Estimation of gestational age in Merino ewes by ultrasound measurement of fetal head size. Crop Pasture Sci. 1989;40(6):1293-1299.

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26(4):239-57.

Khan H, Kusakabe KT, Wakitani S, Hiyama M, Takeshita A, Kiso Y. Expression and localization of NO synthase isoenzymes (iNOS and eNOS) in development of the rabbit placenta. J Reprod Dev. 2012;58(2):231-6.

King JC. Physiology of pregnancy and nutrient metabolism. Am J Clin Nutr. 2000;71(5):1218-1225.

Kirat D, Hamid NEA, Mohamed WE, Hamada M, Shalaby SI. Leptin Gene Expression in Rabbits During Pregnancy and Fetal Life. Int J Biol. 2015;7(2):113.

Kliegman RM. Alterations of fasting glucose and fat metabolism in intrauterine growth-retarded newborn dogs. Am J Physiol. 1989;256(3 Pt 1):E380-5.

Knapik DM, Harris JD, Pangrazzi G, Griesser MJ, Siston RA, Agarwal S, et al. The basic science of continuous passive motion in promoting knee health: a systematic review of studies in a rabbit model. Arthroscopy. 2013;29(10):1722-31.

Kondo A, Li J, Manabe M, Saito K, Kanno T, Maekawa M. Relationship between high-density lipoprotein-cholesterol and malondialdehyde-modified low-density lipoprotein concentrations. J Atheroscler Thromb. 2003;10(2):72-8.

Kostadinov S, Robbins KA, Hayward A. Abnormal TREC-Based Newborn Screening Test in a Premature Neonate with Massive Perivillous Fibrin Deposition of the Placenta. Case Reports Immunol. 2016;2016:5083274.

Krause BJ, Hanson MA, Casanello P. Role of nitric oxide in placental vascular development and function. Placenta. 2011;32(11):797-805.

Krishna U, Bhalerao S. Placental insufficiency and fetal growth restriction. J Obstet Gynaecol India. 2011;61(5):505-11.

Kroener L, Wang ET, Pisarska MD. Predisposing Factors to Abnormal First Trimester Placentation and the Impact on Fetal Outcomes. Semin Reprod Med. 2016;34(1):27-35.

Kurjak A, Predojevic M, Stanojevic M, Kadic AS, Miskovic B, Badreldeen A, et al. Intrauterine growth restriction and cerebral palsy. Acta Inform Med. 2012;18(2):64-82.

Kusinski LC, Stanley JL, Dilworth MR, Hirt CJ, Andersson IJ, Renshall LJ, et al. eNOS knockout mouse as a model of fetal growth restriction with an impaired uterine artery function and placental transport phenotype. Am J Physiol Regul Integr Comp Physiol. 2012;303(1):R86-93.

Lacassie HJ, Germain AM, Valdes G, Fernandez MS, Allamand F, Lopez H. Management of Eisenmenger syndrome in pregnancy with sildenafil and L-arginine. Obstet Gynecol. 2004;103(5 Pt 2):1118-20.

Lackman F, Capewell V, Richardson B, daSilva O, Gagnon R. The risks of spontaneous preterm delivery and perinatal mortality in relation to size at birth according to fetal versus neonatal growth standards. Am J Obstet Gynecol. 2001;184(5):946-53.

Lager S, Powell TL. Regulation of nutrient transport across the placenta. J Pregnancy. 2012;2012:179827.

Lain KY, Catalano PM. Metabolic changes in pregnancy. Clin Obstet Gynecol. 2007;50(4):938-48.

Lambert G, Brichant JF, Hartstein G, Bonhomme V, Dewandre PY. Preeclampsia: an update. Acta Anaesthesiol Belg. 2014;65(4):137-49.

Landon MB, Gabbe SG, Bruner JP, Ludmir J. Doppler umbilical artery velocimetry in pregnancy complicated by insulin-dependent diabetes mellitus. Obstet Gynecol. 1989;73(6):961-5.

Langley-Evans SC, Gardner DS, Jackson AA. Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. J Reprod Fertil. 1996;106(2):307-12.

Larsen JF. Electron microscopy of the chorioallantoic placenta of the rabbit: II. The decidua and the maternal vessels. J Ultrastruc Res. 1963;8(3-4):327-338.

Lecarpentier E, Morel O, Tarrade A, Dahirel M, Bonneau M, Gayat E, et al. Quantification of utero-placental vascularization in a rabbit model of IUGR with three-dimensional power Doppler angiography. Placenta. 2012;33(10):769-75.

Leitich H, Egarter C, Husslein P, Kaider A, Schemper M. A meta-analysis of low dose aspirin for the prevention of intrauterine growth retardation. Br J Obstet Gynaecol. 1997;104(4):450-9.

Lepercq J, Challier JC, Guerre-Millo M, Cauzac M, Vidal H, Hauguel-de Mouzon S. Prenatal leptin production: evidence that fetal adipose tissue produces leptin. J Clin Endocrinol Metab. 2001;86(6):2409-13.

Leveille P, Tarrade A, Dupont C, Larcher T, Dahirel M, Poumerol E, et al. Maternal high-fat diet induces follicular atresia but does not affect fertility in adult rabbit offspring. J Dev Orig Health Dis. 2014;5(2):88-97.

Levene ML, Kornberg J, Williams TH. The incidence and severity of post-asphyxial encephalopathy in full-term infants. Early Hum Dev. 1985;11(1):21-6.

Levy R, Smith SD, Chandler K, Sadovsky Y, Nelson DM. Apoptosis in human cultured trophoblasts is enhanced by hypoxia and diminished by epidermal growth factor. Am J Physiol Cell Physiol. 2000;278(5):C982-8.

Li C, Schlabritz-Loutsevitch NE, Hubbard GB, Han V, Nygard K, Cox LA, et al. Effects of maternal global nutrient restriction on fetal baboon hepatic insulin-like growth factor system genes and gene products. Endocrinology. 2009;150(10):4634-42.

Li L, Jiang Q, Zhang L, Ding G, Gang Zhang Z, Li Q, et al. Angiogenesis and improved cerebral blood flow in the ischemic boundary area detected by MRI after administration of sildenafil to rats with embolic stroke. Brain Res. 2007;1132(1):185-92.

LifeMap Discovery [Internet]. Discovery.lifemapsc.com. 2016 [cited 27 November 2016]. Available from: http://discovery.lifemapsc.com/library/images/early-embryonic-development

Lin TH, Su YN, Shih JC, Hsu HC, Lee CN. Resolution of high uterine artery pulsatility index and notching following sildenafil citrate treatment in a growth-restricted pregnancy. Ultrasound Obstet Gynecol. 2012;40(5):609-10.

Lisle SJ, Lewis RM, Petry CJ, Ozanne SE, Hales CN, Forhead AJ. Effect of maternal iron restriction during pregnancy on renal morphology in the adult rat offspring. Br J Nutr. 2003;90(1):33-9.

Liu Y, Zhang L, Li J, Shan Z, Teng W. Maternal marginal iodine deficiency affects the expression of relative proteins during brain development in rat offspring. J Endocrinol. 2013;217(1):21-9.

Lorenzo PL, Garcia-Garcia RM, Arias-Alvarez M, Rebollar PG. Reproductive and nutritional management on ovarian response and embryo quality on rabbit does. Reprod Domest Anim. 2014;49 Suppl 4:49-55.

Losonczy G, Mucha I, Muller V, Kriston T, Ungvari Z, Tornoci L, et al. The vasoconstrictor effects of L-NAME, a nitric oxide synthase inhibitor, in pregnant rabbits. Br J Pharmacol. 1996;118(4):1012-8.

Lumley J, Watson L, Watson M, Bower C. Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects. Cochrane Database Syst Rev. 2001(3):Cd001056.

Luna RL, Nunes AK, Oliveira AG, Araujo SM, Lemos AJ, Rocha SW, et al. Sildenafil (Viagra(R)) blocks inflammatory injury in LPS-induced mouse abortion: A potential prophylactic treatment against acute pregnancy loss? Placenta. 2015;36(10):1122-9.

Luna RL, Vasconcelos AG, Nunes AK, de Oliveira WH, Barbosa KP, Peixoto CA. Effects of Sildenafil Citrate and Heparin Treatments on Placental Cell Morphology in a Murine Model of Pregnancy Loss. Cells Tissues Organs. 2016;201(3):193-202.

Lyall F, Greer IA, Young A, Myatt L. Nitric oxide concentrations are increased in the feto-placental circulation in intrauterine growth restriction. Placenta. 1996;17(2-3):165-8.

Macari M, Machado CR. Sexual maturity in rabbits defined by the physical and chemical characteristics of the semen. Lab Anim. 1978;12(1):37-9.

Manal AF, Tony MA, Ezzo OH. Feed restriction of pregnant nulliparous rabbit does: consequences on reproductive performance and maternal behaviour. Anim Reprod Sci. 2010;120(1-4):179-86.

Marcorelles P. Placental features in intrauterine growth retardation. J Gynecol Obstet Biol Reprod (Paris). 2013;42(8):996-1007.

Mari G, Hanif F, Kruger M, Cosmi E, Santolaya-Forgas J, Treadwell MC. Middle cerebral artery peak systolic velocity: a new Doppler parameter in the assessment of growth-restricted fetuses. Ultrasound Obstet Gynecol. 2007;29(3):310-6.

Mari G. Middle cerebral artery peak systolic velocity: is it the standard of care for the diagnosis of fetal anemia? J Ultrasound Med. 2005;24(5):697-702.

Marsal K. Obstetric management of intrauterine growth restriction. Best Pract Res Clin Obstet Gynaecol. 2009;23(6):857-870.

Marseille-Tremblay C, Gravel A, Lafond J, Mounier C. Effect of an enriched cholesterol diet during gestation on fatty acid synthase, HMG-CoA reductase and SREBP-1/2 expressions in rabbits. Life Sci. 2007;81(9):772-8.

Marshall VA, Johnson KJ, Moore NP, Rasoulpour RJ, Tornesi B, Carney EW. Comparative response of rat and rabbit conceptuses in vitro to inhibitors of histiotrophic nutrition. Birth Defects Res B Dev Reprod Toxicol. 2015;104(1):1-10.

Marton T, Hargitai B, Bowen C, Cox PM. Elevated brain weight/liver weight ratio in normal body weight centile term perinatal deaths: an indicator of terminal intrauterine malnourishment. Pediatr Dev Pathol. 2013;16(4):267-71.

Matsuoka T, Mizuguchi H, Mizoguchi Y, Endho T, Kamata R, Fukuda K, et al. Effects of restricted feeding on blood parameters in pregnant rabbits. J Toxicol Sci. 2009;34(1):129-37.

Matsuzawa T, Nakata M, Goto I, Tsushima M. Dietary deprivation induces fetal loss and abortion in rabbits. Toxicology. 1981;22(3):255-9.

Mayeur S, Lancel S, Theys N, Lukaszewski MA, Duban-Deweer S, Bastide B, et al. Maternal calorie restriction modulates placental mitochondrial biogenesis and bioenergetic efficiency: putative involvement in fetoplacental growth defects in rats. Am J Physiol Endocrinol Metab. 2013;304(1):E14-22.

McCrabb GJ, Egan AR, Hosking BJ. Maternal undernutrition during mid-pregnancy in sheep: variable effects on placental growth. J Agr Sci. 1992;118(01):127-132.

McDonald TJ, Wu G, Nijland MJ, Jenkins SL, Nathanielsz PW, Jansson T. Effect of 30% nutrient restriction in the first half of gestation on maternal and fetal baboon serum amino acid concentrations. Br J Nutr. 2013;109(8):1382-8.

McNeill BA, Barrell GK, Ridgway MJ, Wellby MP, Prickett TC, Espiner EA. Caloric restriction, but not caloric loading, affects circulating fetal and maternal C-type natriuretic peptide concentrations in late ovine gestation. Reprod Fertil Dev. 2012;24(8):1063-70.

Menchetti L, Brecchia G, Canali C, Cardinali R, Polisca A, Zerani M, et al. Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming. Res Vet Sci. 2015b;98:7-12.

Menchetti L, Brecchia G, Cardinali R, Polisca A, Boiti C. Feed restriction during pregnancy: Effects on body condition and productive performance of primiparous rabbit does. World Rabbit Sci. 2015a;23(1):1-8.

Mergia E, Stegbauer J. Role of Phosphodiesterase 5 and Cyclic GMP in Hypertension. Curr Hypertens Rep. 2016;18(5):39.

Mescher AL. Junqueira's basic histology: text and atlas. 13th ed. Mcgraw-Hill; 2013. 480 p.

Mess A. Placental Evolution within the Supraordinal Clades of Eutheria with the Perspective of Alternative Animal Models for Human Placentation. Adv Biol. 2014;1:21.

Militello M, Pappalardo EM, Ermito S, Dinatale A, Cavaliere A, Carrara S. Obstetric management of IUGR. J Prenat Med. 2009;3(1):6-9.

Miller SL, Loose JM, Jenkin G, Wallace EM. The effects of sildenafil citrate (Viagra) on uterine blood flow and well being in the intrauterine growth-restricted fetus. Am J Obstet Gynecol. 2009;200(1):102.e1-7.

Mitchell ML. Fetal brain to liver weight ratio as a measure of intrauterine growth retardation: analysis of 182 stillborn autopsies. Mod Pathol. 2001;14(1):14-9.

Mizoguchi Y, Matsuoka T, Mizuguchi H, Endoh T, Kamata R, Fukuda K, et al. Changes in blood parameters in New Zealand White rabbits during pregnancy. Lab Anim. 2010;44(1):33-9.

Moce ML, Santacreu MA, Climent A, Blasco A. The effect of divergent selection for uterine capacity on fetal and placental development at term in rabbits: maternal and embryonic genetic effects. J Anim Sci. 2004;82(4):1046-52.

Montoudis A, Boileau S, Simoneau L, Lafond J. Impact of an enriched-cholesterol diet on enzymatic cholesterol metabolism during rabbit gestation. Life Sci. 2003;73(11):1463-77.

Montoudis A, Simoneau L, Brissette L, Forest JC, Savard R, Lafond J. Impact of a cholesterol enriched diet on maternal and fetal plasma lipids and fetal deposition in pregnant rabbits. Life Sci. 1999;64(26):2439-50.

Mossman HW. The rabbit placenta and the problem of placental transmission. Am J Anat. 1923;37(3):433-497.

Motta C, Grosso C, Zanuzzi C, Molinero D, Picco N, Bellingeri R, et al. Effect of Sildenafil on Pre-Eclampsia-Like Mouse Model Induced By L-Name. Reprod Domest Anim. 2015;50(4):611-6.

Mu J, Kanzaki T, Tomimatsu T, Fukuda H, Wasada K, Fujii E, et al. Expression of apoptosis in placentae from mice lacking the prostaglandin F receptor. Placenta. 2002;23(2-3):215-23.

Musial B, Fernandez-Twinn DS, Vaughan OR, Ozanne SE, Voshol P, Sferruzzi-Perri AN, et al. Proximity to Delivery Alters Insulin Sensitivity and Glucose Metabolism in Pregnant Mice. Diabetes. 2016;65(4):851-60.

Myatt L. Placental adaptive responses and fetal programming. J Physiol. 2006;572(Pt 1):25-30.

Naeye RL, Blanc W, Paul C. Effects of maternal nutrition on the human fetus. Pediatrics. 1973;52(4):494-503.

Nafeaa A, Ahmed SA, Fat Hallah S. Effect of Feed Restriction during Pregnancy on Performance and Productivity of New Zealand White Rabbit Does. Vet Med Int. 2011;2011:839737.

Nansseu JR, Fokom-Domgue J, Noubiap JJ, Balti EV, Sobngwi E, Kengne AP. Fructosamine measurement for diabetes mellitus diagnosis and monitoring: a systematic review and meta-analysis protocol. BMJ Open. 2015;5(5):e007689.

Nardozza LM, Araujo Junior E, Barbosa MM, Caetano AC, Lee DJ, Moron AF. Fetal growth restriction: current knowledge to the general Obs/Gyn. Arch Gynecol Obstet. 2012;286(1):1-13.

Nassar AH, Masrouha KZ, Itani H, Nader KA, Usta IM. Effects of sildenafil in Nomega-nitro-L-arginine methyl ester-induced intrauterine growth restriction in a rat model. Am J Perinatol. 2012;29(6):429-34.

Nishimura M. Timing of implantation in New Zealand White rabbits. Congenit Anom. 2001;41(3):198-203.

Nowak C, Joubert M, Jossic F, Masseau A, Hamidou M, Philippe HJ, et al. Perinatal prognosis of pregnancies complicated by placental chronic villitis or intervillositis of unknown etiology and combined lesions: About a series of 178 cases. Placenta. 2016;44:104-8.

Ong KK, Preece MA, Emmett PM, Ahmed ML, Dunger DB. Size at birth and early childhood growth in relation to maternal smoking, parity and infant breast-feeding: longitudinal birth cohort study and analysis. Pediatr Res. 2002;52(6):863-7.

Painter RC, Roseboom TJ, Bleker OP. Prenatal exposure to the Dutch famine and disease in later life: an overview. Reprod Toxicol. 2005;20(3):345-52.

Palinski W, D'Armiento FP, Witztum JL, de Nigris F, Casanada F, Condorelli M, et al. Maternal hypercholesterolemia and treatment during pregnancy influence the long-term progression of atherosclerosis in offspring of rabbits. Circ Res. 2001;89(11):991-6.

Palinski W. Maternal-fetal cholesterol transport in the placenta: good, bad, and target for modulation. Circ Res. 2009;104(5):569-71.

Panda S, Das A, Md Nowroz H. Sildenafil citrate in fetal growth restriction. J Reprod Infertil. 2014;15(3):168-9.

Parraguez VH, Atlagich M, Araneda O, Garcia C, Munoz A, De Los Reyes M, et al. Effects of antioxidant vitamins on newborn and placental traits in gestations at high altitude: comparative study in high and low altitude native sheep. Reprod Fertil Dev. 2011;23(2):285-96.

Parraguez VH, Atlagich M, Diaz R, Cepeda R, Gonzalez C, De los Reyes M, et al. Ovine placenta at high altitudes: comparison of animals with different times of adaptation to hypoxic environment. Anim Reprod Sci. 2006;95(1-2):151-7.

Parraguez VH, Mamani S, Cofre E, Castellaro G, Urquieta B, De Los Reyes M, et al. Disturbances in Maternal Steroidogenesis and Appearance of Intrauterine Growth Retardation at High-Altitude Environments Are Established from Early Pregnancy. Effects of Treatment with Antioxidant Vitamins. PLoS One. 2015;10(11):e0140902.

Parraguez VH, Urquieta B, De los Reyes M, Gonzalez-Bulnes A, Astiz S, Munoz A. Steroidogenesis in sheep pregnancy with intrauterine growth retardation by high-altitude hypoxia: effects of maternal altitudinal status and antioxidant treatment. Reprod Fertil Dev. 2013;25(4):639-45.

Peeters LL, Sheldon RE, Jones MD, Jr., Makowski EL, Meschia G. Blood flow to fetal organs as a function of arterial oxygen content. Am J Obstet Gynecol. 1979;135(5):637-46.

Pellicer B, Herraiz S, Cauli O, Rodrigo R, Asensi M, Cortijo J, et al. Haemodynamic effects of long-term administration of sildenafil in normotensive pregnant and non-pregnant rats. Bjog. 2011;118(5):615-23.

Perry JS. The mammalian fetal membranes. J Reprod Fertil. 1981;62(2):321-35.

Petrere JA, Rohn WR, Grantham LE, 2nd, Anderson JA. Food restriction during organogenesis in rabbits: effects on reproduction and the offspring. Fundam Appl Toxicol. 1993;21(4):517-22.

Picciano MF. Pregnancy and lactation: physiological adjustments, nutritional requirements and the role of dietary supplements. J Nutr. 2003;133(6):1997s-2002s.

Picone O, Laigre P, Fortun-Lamothe L, Archilla C, Peynot N, Ponter AA, et al. Hyperlipidic hypercholesterolemic diet in prepubertal rabbits affects gene expression in the embryo, restricts fetal growth and increases offspring susceptibility to obesity. Theriogenology. 2011;75(2):287-99.

Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. Placenta. 2006;27(9-10):939-58.

Pisaneschi S, Strigini FA, Sanchez AM, Begliuomini S, Casarosa E, Ripoli A, et al. Compensatory feto-placental upregulation of the nitric oxide system during fetal growth restriction. PLoS One. 2012;7(9):e45294.

Polisca A, Scotti L, Orlandi R, Brecchia G, Boiti C. Doppler evaluation of maternal and fetal vessels during normal gestation in rabbits. Theriogenology. 2010;73(3):358-66.

Pomorski M, Zimmer M, Florjanski J, Michniewicz J, Wiatrowski A, Fuchs T, et al. Comparative analysis of placental vasculature and placental volume in normal and IUGR pregnancies with the use of three-dimensional Power Doppler. Arch Gynecol Obstet. 2012;285(2):331-7.

Popjak G. Maternal and foetal tissue- and plasma-lipids in normal and cholesterol-fed rabbits. J Physiol. 1946;105(3):236-54.2.

Poudel R, McMillen IC, Dunn SL, Zhang S, Morrison JL. Impact of chronic hypoxemia on blood flow to the brain, heart, and adrenal gland in the late-gestation IUGR sheep fetus. Am J Physiol Regul Integr Comp Physiol. 2015;308(3):R151-62.

Purcell TL, Buhimschi IA, Given R, Chwalisz K, Garfield RE. Inducible nitric oxide synthase is present in the rat placenta at the fetal-maternal interface and decreases prior to labour. Mol Hum Reprod. 1997;3(6):485-91.

Purcell TL, Given R, Chwalisz K, Garfield RE. Nitric oxide synthase distribution during implantation in the mouse. Mol Hum Reprod. 1999;5(5):467-75.

Pyriochou A, Zhou Z, Koika V, Petrou C, Cordopatis P, Sessa WC, et al. The phosphodiesterase 5 inhibitor sildenafil stimulates angiogenesis through a protein kinase G/MAPK pathway. J Cell Physiol. 2007;211(1):197-204.

Ramesar SV, Mackraj I, Gathiram P, Moodley J. Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague-Dawley rats. Eur J Obstet Gynecol Reprod Biol. 2010;149(1):22-6.

Ramsey EM, Harris JW. Comparison of uteroplacental vasculature and circulation in the rhesus monkey and man. 1st ed. Carnegie Institution of Washington; 1966. 70 p.

Ravipati G, McClung JA, Aronow WS, Peterson SJ, Frishman WH. Type 5 phosphodiesterase inhibitors in the treatment of erectile dysfunction and cardiovascular disease. Cardiol Rev. 2007;15(2):76-86.

Renshall L. Antenatal Sildenafil Citrate Treatment in a Mouse Model of Fetal Growth Restriction: Effects on Fetus and Offspring [doctoral thesis]. [Manchester (UK)]: University of Manchester; 2015.

Resende TP, Andrade RP, Palmeirim I. Timing embryo segmentation: dynamics and regulatory mechanisms of the vertebrate segmentation clock. Biomed Res Int. 2014;2014:718683.

Roberts AB, Baker JR, Court DJ, James AG, Henley P, Ronayne ID. Fructosamine in diabetic pregnancy. Lancet. 1983;2(8357):998-1000.

Rommers JM, Meijerhof R, Noordhuizen JP, Kemp B. The effect of level of feeding in early gestation on reproductive success in young rabbit does. Anim Reprod Sci. 2004;81(1-2):151-8.

Ropacka-Lesiak M, Korbelak T, Swider-Musielak J, Breborowicz G. Cerebroplacental ratio in prediction of adverse perinatal outcome and fetal heart rate disturbances in uncomplicated pregnancy at 40 weeks and beyond. Arch Med Sci. 2015;11(1):142-8.

Rosati P, Exacoustos C, Puggioni GF, Mancuso S. Growth retardation in pregnancy: experimental model in the rabbit employing electrically induced thermal placental injury. Int J Exp Pathol. 1995;76(3):179-81.

Roseboom TJ, Painter RC, de Rooij SR, van Abeelen AF, Veenendaal MV, Osmond C, et al. Effects of famine on placental size and efficiency. Placenta. 2011;32(5):395-9.

Rossant J, Cross JC. Placental development: lessons from mouse mutants. Nat Rev Genet. 2001;2(7):538-48.

Roth I, Corry DB, Locksley RM, Abrams JS, Litton MJ, Fisher SJ. Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. J Exp Med. 1996;184(2):539-48.

Russell JK. Early teenage pregnancy. [Edinburgh (UK)]: Churchill Livingstone; 1982.

Rutherford JN. Toward a nonhuman primate model of fetal programming: phenotypic plasticity of the common marmoset fetoplacental complex. Placenta. 2012;33 Suppl 2:e35-9.

Ryan EA, Enns L. Role of gestational hormones in the induction of insulin resistance. J Clin Endocrinol Metab. 1988;67(2):341-7.

Sadler TW. Langman's medical embryology. 12th ed. Lippincott Williams & Wilkins; 2011. 400 p.

Sanchez-Aparicio P, Mota-Rojas D, Nava-Ocampo AA, Trujillo-Ortega ME, Alfaro-Rodriguez A, Arch E, et al. Effects of sildenafil on the fetal growth of guinea pigs and their ability to survive induced intrapartum asphyxia. Am J Obstet Gynecol. 2008;198(1):127.e1-6.

Sandovici I, Hoelle K, Angiolini E, Constancia M. Placental adaptations to the maternal-fetal environment: implications for fetal growth and developmental programming. Reprod Biomed Online. 2012;25(1):68-89.

Sankaran S, Kyle PM. Aetiology and pathogenesis of IUGR. Best Pract Res Clin Obstet Gynaecol. 2009;23(6):765-77.

Satterfield MC, Bazer FW, Spencer TE, Wu G. Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. J Nutr. 2010;140(2):251-8.

Schiessl B, Mylonas I, Hantschmann P, Kuhn C, Schulze S, Kunze S, et al. Expression of endothelial NO synthase, inducible NO synthase, and estrogen receptors alpha and beta in placental tissue of normal, preeclamptic, and intrauterine growth-restricted pregnancies. J Histochem Cytochem. 2005;53(12):1441-9.

Schroder HJ. Models of fetal growth restriction. Eur J Obstet Gynecol Reprod Biol. 2003;110 Suppl 1:S29-39.

Schulz LC, Schlitt JM, Caesar G, Pennington KA. Leptin and the placental response to maternal food restriction during early pregnancy in mice. Biol Reprod. 2012;87(5):120.

Schulz LC. The Dutch Hunger Winter and the developmental origins of health and disease. Proc Natl Acad Sci U S A. 2010;107(39):16757-8.

Seidel GE, Bowen RA, Kane MT. In vitro fertilization, culture, and transfer of rabbit ova. Fertil Steril. 1976;27(7):861-70.

Serrano NC, Casas JP, Diaz LA, Paez C, Mesa CM, Cifuentes R, et al. Endothelial NO synthase genotype and risk of preeclampsia: a multicenter case-control study. Hypertension. 2004;44(5):702-7.

Sferruzzi-Perri AN, Camm EJ. The Programming Power of the Placenta. Front Physiol. 2016;7:33.

Sferruzzi-Perri AN, Lopez-Tello J, Fowden AL, Constancia M. Maternal and fetal genomes interplay through phosphoinositol 3-kinase(PI3K)-p110alpha signaling to modify placental resource allocation. Proc Natl Acad Sci U S A. 2016;113(40):11255-60.

Sferruzzi-Perri AN, Vaughan OR, Coan PM, Suciu MC, Darbyshire R, Constancia M, et al. Placental-specific Igf2 deficiency alters developmental adaptations to undernutrition in mice. Endocrinology. 2011;152(8):3202-12.

Shah PS, Shah V. Influence of the maternal birth status on offspring: a systematic review and meta-analysis. Acta Obstet Gynecol Scand. 2009;88(12):1307-18.

Sharp AN, Heazell AE, Crocker IP, Mor G. Placental apoptosis in health and disease. Am J Reprod Immunol. 2010;64(3):159-69.

Sibley CP, Brownbill P, Dilworth M, Glazier JD. Review: Adaptation in placental nutrient supply to meet fetal growth demand: implications for programming. Placenta. 2010;31 Suppl:S70-4.

Sibley CP, Turner MA, Cetin I, Ayuk P, Boyd CA, D'Souza SW, et al. Placental phenotypes of intrauterine growth. Pediatr Res. 2005;58(5):827-32.

Simitzis PE, Symeon GK, Kominakis AP, Bizelis IA, Chadio SE, Abas Z, et al. Severe maternal undernutrition and post-weaning behavior of rabbits. Physiol Behav. 2015;141:172-9.

Simoes RV, Munoz-Moreno E, Carbajo RJ, Gonzalez-Tendero A, Illa M, Sanz-Cortes M, et al. In Vivo Detection of Perinatal Brain Metabolite Changes in a Rabbit Model of Intrauterine Growth Restriction (IUGR). PLoS One. 2015;10(7):e0131310.

Simoes RV, Munoz-Moreno E, Cruz-Lemini M, Eixarch E, Bargallo N, Sanz-Cortes M, et al. Brain metabolite alterations in infants born preterm with intrauterine growth restriction: association with structural changes and neurodevelopmental outcome. Am J Obstet Gynecol. 2016.

Simoni G, Sirchia SM. Confined placental mosaicism. Prenat Diagn. 1994;14(13):1185-9.

Sivan E, Boden G. Free fatty acids, insulin resistance, and pregnancy. Curr Diab Rep. 2003;3(4):319-22.

Smith SC, Baker PN, Symonds EM. Increased placental apoptosis in intrauterine growth restriction. Am J Obstet Gynecol. 1997;177(6):1395-401.

Snoeck A, Remacle C, Reusens B, Hoet JJ. Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate. 1990;57(2):107-18.

Soliman AT, Eldabbagh M, Saleem W, Zahredin K, Shatla E, Adel A. Placental weight: relation to maternal weight and growth parameters of full-term babies at birth and during childhood. J Trop Pediatr. 2013;59(5):358-64.

Solmi F, Sallis H, Stahl D, Treasure J, Micali N. Low birth weight in the offspring of women with anorexia nervosa. Epidemiol Rev. 2014;36:49-56.

Soni S, Rath G, Prasad CP, Salhan S, Saxena S, Jain AK. Apoptosis and Bcl-2 protein expression in human placenta over the course of normal pregnancy. Anat Histol Embryol. 2010;39(5):426-31.

Sparks JW, Cetin I. Determinants of intrauterine growth. In: Hay WW, editor. Neonatal Nutrition and Metabolism, Cambridge University Press; 2006. pp. 23-31.

Stanley JL, Andersson IJ, Hirt CJ, Moore L, Dilworth MR, Chade AR, et al. Effect of the anti-oxidant tempol on fetal growth in a mouse model of fetal growth restriction. Biol Reprod. 2012a;87(1):25, 1-8.

Stanley JL, Andersson IJ, Poudel R, Rueda-Clausen CF, Sibley CP, Davidge ST, et al. Sildenafil citrate rescues fetal growth in the catechol-O-methyl transferase knockout mouse model. Hypertension. 2012b;59(5):1021-8.

Stanley JL, Sulek K, Andersson IJ, Davidge ST, Kenny LC, Sibley CP, et al. Sildenafil Therapy Normalizes the Aberrant Metabolomic Profile in the Comt(-/-) Mouse Model of Preeclampsia/Fetal Growth Restriction. Sci Rep. 2015;5:18241.

Su RW, Fazleabas AT. Implantation and Establishment of Pregnancy in Human and Nonhuman Primates. In: Geisert RD, Bazer FW, editors. Regulation of Implantation and Establishment of Pregnancy in Mammals. Springer International Publishing; 2015. pp. 189-213.

Suhag A, Berghella V. Intrauterine growth restriction (IUGR): etiology and diagnosis. Curr Obstet Gynecol Rep. 2013;2(2):102-111.

Sullivan AD, Nyirenda T, Cullinan T, Taylor T, Harlow SD, James SA, et al. Malaria infection during pregnancy: intrauterine growth retardation and preterm delivery in Malawi. J Infect Dis. 1999;179(6):1580-3.

Sun X, Wang K, Wang W, Li B. Clinical study on sildenafil in treatment of pregnant women with pulmonary arterial hypertension. Zhonghua Fu Chan Ke Za Zhi. 2014;49(6):414-8.

Susser E, St Clair D. Prenatal famine and adult mental illness: interpreting concordant and discordant results from the Dutch and Chinese Famines. Soc Sci Med. 2013;97:325-30.

Swanson AM, David AL. Animal models of fetal growth restriction: Considerations for translational medicine. Placenta. 2015;36(6):623-30.

Sweeting JN, Wells PG. New Zealand white rabbit progeny exposed in utero to methanol are resistant to skeletal anomalies reported for rodents, but exhibit a novel vertebral defect. Reprod Toxicol. 2015;58:104-10.

Symeon GK, Goliomytis M, Bizelis I, Papadomichelakis G, Pagonopoulou O, Abas Z, et al. Effects of gestational maternal undernutrition on growth, carcass composition and meat quality of rabbit offspring. PLoS One. 2015;10(2):e0118259.

Tan S, Drobyshevsky A, Jilling T, Ji X, Ullman LM, Englof I, et al. Model of cerebral palsy in the perinatal rabbit. J Child Neurol. 2005;20(12):972-9.

Tan S, Liu YY, Nielsen VG, Skinner K, Kirk KA, Baldwin ST, et al. Maternal infusion of antioxidants (Trolox and ascorbic acid) protects the fetal heart in rabbit fetal hypoxia. Pediatr Res. 1996;39(3):499-503.

Tan S, Zhou F, Nielsen VG, Wang Z, Gladson CL, Parks DA. Increased injury following intermittent fetal hypoxia-reoxygenation is associated with increased free radical production in fetal rabbit brain. J Neuropathol Exp Neurol. 1999;58(9):972-81.

Tan S, Zhou F, Nielsen VG, Wang Z, Gladson CL, Parks DA. Sustained hypoxia-ischemia results in reactive nitrogen and oxygen species production and injury in the premature fetal rabbit brain. J Neuropathol Exp Neurol. 1998;57(6):544-53.

Tarazona JV, Rodriguez C, Alonso E, Saez M, Gonzalez F, San Andres MD, et al. Toxicokinetics of perfluorooctane sulfonate in rabbits under environmentally realistic exposure conditions and comparative assessment between mammals and birds. Toxicol Lett. 2016;241:200-6.

Tarrade A, Lecarpentier E, Gil S, Morel O, Zahr N, Dahirel M, et al. Analysis of placental vascularization in a pharmacological rabbit model of IUGR induced by L-NAME, a nitric oxide synthase inhibitor. Placenta. 2014;35(4):254-9.

Tarrade A, Rousseau-Ralliard D, Aubriere MC, Peynot N, Dahirel M, Bertrand-Michel J, et al. Sexual dimorphism of the feto-placental phenotype in response to a high fat and control maternal diets in a rabbit model. PLoS One. 2013;8(12):e83458.

Thakur A, Sase M, Lee JJ, Thakur V, Buchmiller TL. Ontogeny of insulin-like growth factor 1 in a rabbit model of growth retardation. J Surg Res. 2000;91(2):135-40.

Tikoo K, Patel G, Kumar S, Karpe PA, Sanghavi M, Malek V, et al. Tissue specific up regulation of ACE2 in rabbit model of atherosclerosis by atorvastatin: role of epigenetic histone modifications. Biochem Pharmacol. 2015;93(3):343-51.

Toal M, Chan C, Fallah S, Alkazaleh F, Chaddha V, Windrim RC, et al. Usefulness of a placental profile in high-risk pregnancies. Am J Obstet Gynecol. 2007;196(4):363.e1-7.

Torre I, Gonzalez-Tendero A, Garcia-Canadilla P, Crispi F, Garcia-Garcia F, Bijnens B, et al. Permanent cardiac sarcomere changes in a rabbit model of intrauterine growth restriction. PLoS One. 2014;9(11):e113067.

Torres-Rovira L, Tarrade A, Astiz S, Mourier E, Perez-Solana M, de la Cruz P, et al. Sex and breed-dependent organ development and metabolic responses in foetuses from lean and obese/leptin resistant swine. PLoS One. 2013;8(7):e66728.

Trapani A, Jr., Goncalves LF, Trapani TF, Franco MJ, Galluzzo RN, Pires MM. Comparison between transdermal nitroglycerin and sildenafil citrate in intrauterine growth restriction: effects on uterine, umbilical and fetal middle cerebral artery pulsatility indices. Ultrasound Obstet Gynecol. 2016;48(1):61-5.

Triunfo S, Lanzone A. Impact of maternal under nutrition on obstetric outcomes. J Endocrinol Invest. 2015;38(1):31-8.

Uvebrant P, Hagberg G. Intrauterine growth in children with cerebral palsy. Acta Paediatr. 1992;81(5):407-12.

Uzan S, Beaufils M, Breart G, Bazin B, Capitant C, Paris J. Prevention of fetal growth retardation with low-dose aspirin: findings of the EPREDA trial. Lancet. 1991;337(8755):1427-31.

Vaag AA, Grunnet LG, Arora GP, Brons C. The thrifty phenotype hypothesis revisited. Diabetologia. 2012;55(8):2085-8.

Valentino SA, Tarrade A, Aioun J, Mourier E, Richard C, Dahirel M, et al. Maternal exposure to diluted diesel engine exhaust alters placental function and induces intergenerational effects in rabbits. Part Fibre Toxicol. 2016;13(1):39.

van Vliet E, Eixarch E, Illa M, Arbat-Plana A, Gonzalez-Tendero A, Hogberg HT, et al. Metabolomics reveals metabolic alterations by intrauterine growth restriction in the fetal rabbit brain. PLoS One. 2013;8(5):e64545.

Vasiljevic N, Vasiljevic M, Plecas D. [The role of nutritional factors in pre-eclampsia and eclampsia]. Srp Arh Celok Lek. 1996;124(5-6):156-9.

Venkatesh KK, Lurie MN, Triche EW, De Bruyn G, Harwell JI, McGarvey ST, et al. Growth of infants born to HIV-infected women in South Africa according to maternal and infant characteristics. Trop Med Int Health. 2010;15(11):1364-74.

Villar J, Cogswell M, Kestler E, Castillo P, Menendez R, Repke JT. Effect of fat and fat-free mass deposition during pregnancy on birth weight. Am J Obstet Gynecol. 1992;167(5):1344-52.

Wang Y. Vascular Biology of the Placenta. Colloquium Series on Integrated Systems Physiology: From Molecule to Function. 2010;2(1):1-98.

Watkins AJ, Lucas ES, Marfy-Smith S, Bates N, Kimber SJ, Fleming TP. Maternal nutrition modifies trophoblast giant cell phenotype and fetal growth in mice. Reproduction. 2015;149(6):563-75.

Wei B, Mao F, Guo Y, Yao Q, Tang C, Xu Y, et al. Using 7.0T MRI T2 mapping to detect early changes of the cartilage matrix caused by immobilization in a rabbit model of immobilization-induced osteoarthritis. Magn Reson Imaging. 2015;33(8):1000-6.

Weiner CP. Pathogenesis, evaluation, and potential treatments for severe, early onset growth retardation. Semin Perinatol. 1989;13(4):320-7.

Weisbroth SH, Flatt RE, Kraus AL. The biology of the laboratory rabbit. 1st ed. Academic Press; 1974. 510 p.

Wells MY, Decobecq CP, Decouvelaere DM, Justice C, Guittin P. Changes in clinical pathology parameters during gestation in the New Zealand white rabbit. Toxicol Pathol. 1999;27(3):370-9.

Wilkins-Haug L, Roberts DJ, Morton CC. Confined placental mosaicism and intrauterine growth retardation: a case-control analysis of placentas at delivery. Am J Obstet Gynecol. 1995;172(1 Pt 1):44-50.

Williamson CS. Nutrition in pregnancy. Nutr Bull. 2006;31(1):28-59.

Wooding P, Burton G. Haemochorial placentation: mouse, rabbit, man, apes, monkeys. In: Wooding P, Burton G, editors. Comparative Placentation: Structures, Functions and Evolution. Springer-Verlag Berlin Heidelberg; 2008. pp. 185-230.

Woollett LA. The origins and roles of cholesterol and fatty acids in the fetus. Curr Opin Lipidol. 2001;12(3):305-12.

World Health Organization (WHO), UNICEF. Low birthweight. Country, regional and global estimates. New York: UNICEF. 2004:1-4.

Wu G, Bazer FW, Wallace JM, Spencer TE. Board-invited review: intrauterine growth retardation: implications for the animal sciences. J Anim Sci. 2006;84(9):2316-37.

Xiccato G, Parigi-Bini R, Dalle Zotte A, Carazzolo A, Cossu ME. Effect of dietary energy level, addition of fat and physiological state on performance and energy balance of lactating and pregnant rabbit does. Anim Sci. 1995;61(02):387-398.

Xu K, Dong M, Zhou J. Changes of maternal and umbilical serum nitric oxide in patients with the intrauterine growth retardation. Zhonghua Fu Chan Ke Za Zhi. 2000;35(12):715-6.

Yallampalli C, Garfield RE. Inhibition of nitric oxide synthesis in rats during pregnancy produces signs similar to those of preeclampsia. Am J Obstet Gynecol. 1993;169(5):1316-20.

Yanni AE. The laboratory rabbit: an animal model of atherosclerosis research. Lab Anim. 2004;38(3):246-56.

Zhang R, Wang Y, Zhang L, Zhang Z, Tsang W, Lu M, et al. Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. Stroke. 2002;33(11):2675-80.

Zhang S, Regnault TR, Barker PL, Botting KJ, McMillen IC, McMillan CM, et al. Placental adaptations in growth restriction. Nutrients. 2015;7(1):360-89.

Zhou X, Dong J, Zhang L, Liu J, Dong X, Yang Q, et al. Hyperglycemia has no effect on development of restenosis after percutaneous transluminal angioplasty (PTA) in a diabetic rabbit model. J Endocrinol. 2015;224(2):119-25.

Zilversmit DB, Remington M, Hughes LB. Fetal growth and placental permeability in rabbits fed cholesterol. J Nutr. 1972;102(12):1681-7.

Zone MA, Wanke MM. Diagnosis of canine fetal health by ultrasonography. J Reprod Fertil Suppl. 2001;57:215-9.

Curriculum Vitae

Jorge López Tello

Date and Place of birth: 03rd August 1990, Madrid, Spain
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Education

2014-current	**PhD candidate** Complutense University of Madrid. Spain
2013-2014	**Official master's degree in animal health and production** Complutense University of Madrid. Spain
2008-2013	**Bachelor degree in veterinary medicine** Alfonso X El Sabio University, Madrid. Spain

Research interests & personal statement

During my PhD I have chosen rabbits as the animal model not only to help me understand the pathogenesis of complicated pregnancies but also and more specifically, to determine the consequences of reduced maternal food intake and IUGR. Moreover, I have investigated the effects of a pharmacological therapy based on the administration of Sildenafil Citrate to counteract placental insufficiency and IUGR.

Despite my short research career, I have also been involved in other projects, i.e. rabbit oocyte studies to knock out models in rodents. This particular project was conducted by the University of Cambridge, in Dr. Sferruzzi-Perri laboratory at the Centre for Trophoblast Research (CTR). Using genetic tools, we found that altering the expression of a growth gene (*Igf2*) in only the hormone-producing part of the mouse placenta, altered placenta development and the mother´s ability to properly adapt her body and metabolism during pregnancy.

My future career plan is to specialise in determining the specific and fascinating role of placental hormone when it comes to adapting maternal metabolism to favour nutrient allocation to fetal growth. I hope I will obtain a fellowship that will help me address these professional goals. It will also provide me with further training possibilities to study *in-vivo* fetoplacental physiology and endocrinology as well as different *in-vitro* techniques.

Research funding & scholarships

1) **COST SALAAM.** Mobility grant
   Dr. Sferruzzi-Perri lab; University of Cambridge; Jan-March 2017
2) **COST EPICONCEPT Meeting**
   Congress assistance; Sep 2016
3) **Multi-scale Imaging for the Study of Reproduction**
   Satellite Course. INRA. France; June 2016

4) **COST SALAAM Meeting**
Congress assistance; Dec 2015

5) **COST EPICONCEPT**. Mobility grant
Dr. Sferruzzi-Perri lab; University of Cambridge; Jan-April 2016

6) **Erasmus Placement II**. EU Programme for Lifelong Learning. Mobility grant
Dr. Sferruzzi-Perri lab; University of Cambridge; Oct-Dec 2015

7) **Erasmus Placement I**. EU Programme for Lifelong Learning. Mobility grant
Dr. Sferruzzi-Perri lab; University of Cambridge; Jun-Sep 2015

8) **Erasmus Exchange Student.** Mobility grant
Veterinary Faculty; University of Bologna (Unibo). Italy; 2011-2012

Awards

Imanova Best Speaker Prize 2016. Best PhD Student Oral Communication: "The role of H19/Igf2 imprinted locus in regulating maternal-fetal resource allocation in mouse pregnancy". Annual PDN Symposium, University of Cambridge, UK

Publications (Indexed publications in JCR)

1) Decato BE, **López-Tello J**, Sferruzzi-Perri AN, Smith AD, Dean MD. (2016) DNA methylation divergence and tissue specialization in the developing mouse placenta. *Molecular Biology and Evolution*. Provisionally accepted pending final revision (ID: MBE-16-0603) **(Impact Factor: 13.649, Q1)**.

2) **López-Tello J**, Arias-Álvarez M, Martínez-Jiménez MA, García-García RM, Rodríguez M, Lorenzo PL, Bermejo-Poza R, González-Bulnes A, Rebollar PG. (2016) Competition for materno-fetal resource partitioning in a rabbit model of undernourished pregnancy. *Plos One*. Provisionally accepted pending final revision (ID: PONE-D-16-24553) **(Impact Factor: 3.057, Q1)**.

3) Arias-Álvarez M, García-García RM, **López-Tello J**, Rebollar PG, Gutiérrez-Adán A, Lorenzo PL. (2016) In vivo and in vitro maturation of rabbit oocyte differently affects the gene expression profile, mitochondrial distribution, apoptosis and early embryo development. *Reproduction, Fertility and Development*. doi: 10.1071/RD15553 **(Impact Factor: 2.135, Q1)**.

4) Sferruzzi-Perri AN, **López-Tello J**, Fowden A, Constancia M. (2016) Maternal and fetal genomes interplay through PI3K-p110α signalling to modify placental resource allocation. *Proceedings of the National Academy of Sciences*, *113*(40), 11255-11260. doi: 10.1073/pnas.1602012113 **(Impact Factor: 9.423, Q1)**.

5) **López-Tello J**, Arias-Álvarez M, Martínez-Jiménez MA, Barbero-Fernández A, García-García RM, Rodríguez M, Lorenzo PL, Torres-Rovira L, Astiz S, González-Bulnes A, Rebollar PG. (2016) The effects of sildenafil citrate on fetoplacental development and hemodynamics in a rabbit model of intrauterine growth restriction. *Reproduction, Fertility and Development*. doi: 10.1071/RD15330 **(Impact Factor: 2.135, Q1)**.

6) **López-Tello J**, Barbero-Fernández A, González-Bulnes A, Astiz S, Rodríguez M, Formoso-Rafferty N, Arias-Álvarez M, Rebollar PG. (2015) Characterization of early changes in fetoplacental hemodynamics in a diet-induced rabbit model of IUGR**.** *Journal of Developmental Origins of Health and Disease*, *2015, vol. 6, no 05, p. 454-461.* doi: 10.1017/S2040174415001385 **(Impact Factor: 1.733, Q2)**.

Abstracts (Presenter; *Oral exposition; †Poster exposition)

1) **López-Tello J***, Sferruzzi-Perri AN. Determining the role of placental endocrine zone Igf2 in the "tug of war" over resources between mother and fetus in mice. EPICONCEPT Conference, Sicily, Italy 2016.

2) **López-Tello J***, Sferruzzi-Perri AN. The role of H19/Igf2 imprinted locus in regulating maternal-fetal resource allocation in mouse pregnancy. Annual Symposium of the Department of Physiology, Development and Neuroscience. University of Cambridge, UK 2016.

3) Sferruzzi-Perri AN*, **López-Tello J**, Fowden A, Constancia M. Maternal and fetal genomes interplay through phosphoinositol 3-kinase (PI3K)-p110α signalling to modify placental resource allocation to fetal growth. FNPS Congress, Cambridge, UK 2016.

4) **López-Tello J**, Sferruzzi-Perri AN†. The mouse dam fails to metabolically adapt to the pregnant state in response to a deficiency of Igf2 in placental endocrine cells. FNPS Congress, Cambridge, UK 2016.

5) Arias-Álvarez M†, García-García RM, **López-Tello J**, Nieto K, Rebollar PG, Gutiérrez-Adán A, Lorenzo PL. Alpha-tocopherol affects gene expression patterns of rabbit cumulus-oocyte complexes and reduces the apoptosis rate during *in vitro* maturation. AETE Congress, Barcelona, Spain 2016.

6) Arias-Álvarez M, García-García RM, **López-Tello J**†, Gutiérrez-Adán A, Rebollar PG, Lorenzo PL. Cellular and molecular markers of oocyte quality: differences between immature, in vivo and in vitro-maturation in the rabbit model. ICAR Congress. Tours, France 2016.

7) Rodríguez M, Febrel N, **López-Tello J**, García-García RM, Arias-Álvarez M, Millán P, Formoso-Rafferty N, Lorenzo PL, Rebollar PG†. Preimplantational study in rabbit does supplemented with n-3 polyunsaturated fatty acids. The 11th World Rabbit Congress. Qingdao, China 2016.

8) Rodríguez M*, **López-Tello J**, Arias-Álvarez M, García-García RM, Formoso-Rafferty N, Lorenzo PL, Rebollar PG. Fetoplacental and organ development in foetuses of rabbit does supplemented with n-3 PUFA during pregnancy. The 11th World Rabbit Congress. Qingdao, China 2016.

9) **López-Tello J**, Sferruzzi-Perri AN†. The role of Igf2 in regulating maternal-fetal resource allocation in mouse pregnancy. IUPS Congress. Dublin, Ireland 2016.

10) Rodríguez M†, Febrel N, **López-Tello J**, Formoso-Rafferty N, Millan P, García-García RM, Arias-Álvarez M, Lorenzo PL, Rebollar PG. La suplementación de los piensos de las conejas con EPA y DHA mejora el perfil insaturado de los ácidos grasos de la leche y sus parámetros reproductivos. Jornadas MEDGAN. Madrid, Spain 2016.

11) **López-Tello J**†, Arias-Álvarez M, Martínez-Jiménez MA, Barbero-Fernández A, García-García RM, Rodríguez M, Lorenzo PL, Torres-Rovira L, Astiz S, González-Bulnes A, Rebollar PG. Feto-placental development and hemodynamics can be modulated by sildenafil citrate administration in a rabbit model of intrauterine growth restriction. SALAAM Conference. Poznan, Poland 2015.

12) **López-Tello J**, Arias-Álvarez M†, Martínez-Jiménez MA, García-García RM, Rodríguez M, Lorenzo PL, Rebollar PG. Feto-Placental development in the rabbit model can be altered by maternal food restriction from early pregnancy. EPICONCEPT Conference. Crete, Greece 2015.

13) Sferruzzi-Perri AN†, Khaira J, Kusinski L, Sandovici I, **López-Tello J**, Higgins J, Fowden AL, Constancia M. The role of the phosphoinositol kinase (PI3K) p110α in regulating placental phenotype and fetal growth. CTR Annual Trophoblast Meeting. Cambridge, UK 2015.

14) **López-Tello J**, Rodríguez M, Formoso-Rafferty N, Bermejo R, García-García RM, Arias-Álvarez M, Lorenzo PL, Rebollar PG†. Intrauterine position affects correct foetal-placental development in the rabbit. ASESCU Congress. Santiago de Compostela, Spain 2015.

15) Rodríguez M*, **López-Tello J**, Formoso-Rafferty N, García-García RM, Arias-Álvarez M, Lorenzo PL, Rebollar PG. Diets supplemented with polyunsaturated fatty acids n-3 improve foetal-placental development of rabbit does. ASESCU Congress. Santiago de Compostela, Spain 2015.

16) Febrel N*, Rodríguez M, Velasco B, **López-Tello J**, García-García RM, Arias-Álvarez M, Lorenzo PL, Rebollar PG. Effect of litter size and polyunsaturated fatty acid n-3 supplementation in lactating rabbit does. ASESCU Congress. Santiago de Compostela, Spain 2015.

17) Febrel N*, Rodríguez M, **López-Tello J**, Velasco B, Millan P, García-García RM, Arias-Álvarez M, Lorenzo PL, Rebollar PG. Diets enriched with polyunsaturated fatty acids n-3 improve rabbit does fertility, kits dimensions and increase progesterone concentrations. ITEA Congress. Zaragoza, Spain 2015.

18) **López-Tello J***, Arias-Álvarez M, García-García RM, Rodríguez M, Formoso-Rafferty N, Bermejo R, Lorenzo PL, Rebollar PG. The effect of different maternal undernutrition protocols on the fetoplacental development in rabbit does. ITEA Congress. Zaragoza, Spain 2015.

19) Rodríguez M*, Velasco B, **López-Tello J**, García-García RM, Arias-Álvarez M, Lorenzo PL, Rebollar PG. Effect of different feed strategies and oestrus synchronization in rabbit does. ITEA Congress. Zaragoza, Spain 2015.

20) **López-Tello J***, Arias-Alvarez M, Gonzalez-Bulnes A, Astiz S, García-García RM, Rodríguez M, Lorenzo PL, Rebollar PG. 91 Sildenafil citrate modifies fetoplacental development in a rabbit model of intrauterine growth restriction. IETS Congress. Versalles, France 2015.

21) **López-Tello J***, Arias-Álvarez M. Efecto de la nutrición materna sobre el desarrollo fetal y placentario en la oveja. VII Congreso de Estudiantes de Ciencia, Tecnología e Ingeniería Agronómica. Universidad Politécnica de Madrid. Spain 2015.

22) **López-Tello J**†**,** Barbero-Fernández A, Aguado E, Ausín L, Arias-Álvarez M, González-Bulnes A, Rebollar PG. Estudio del desarrollo fetal y placentario mediante Ultrasonografía y Doppler color en la coneja. Congreso de Estudiantes Universidad Complutense de Madrid. Spain 2014.

23) **López-Tello J**, Arias-Álvarez M, Rebollar PG. Estudio del retraso en el crecimiento del feto y de la placenta en la coneja. I Semana de la Biotecnología en la Universidad Politécnica de Madrid. Spain 2014.

Invited talks

19–02–2015 **"Sildenafil citrate modifies fetoplacental development in a rabbit model of intrauterine growth restriction"**

Centre for Trophoblast Research. Department of Physiology, Development and Neuroscience. University of Cambridge, UK

Public Outreach

1) XVI Semana de la Ciencia "Ven a manipular gametos y embriones a nuestro laboratorio"

I was involved in a workshop organized to undergraduates about different laboratory techniques involving embryo recovery and physiology of the rabbit gestation. This workshop was hold in the Veterinary Faculty of the University Complutense of Madrid on the 25th of November 2016.

2) **López-Tello J**, Arias-Álvarez M, Rebollar PG. (2016) Restricción alimentaria durante la gestación en conejas. ¿Existe un conflicto de intereses entre madre y feto? *Boletín de cunicultura lagomorpha*, (179), 14-19. *(Publication)*

Membership of scientific societies

2016 **The Physiological Society UK**

2016 **The International Society of Developmental Origins of Health and Disease**

Additional courses

1) **Radioactivity Substances: An introduction to Suitable and Safe Use Course**. Department of Biochemistry, University of Cambridge, UK 2016.
2) **Placental Disease: Intrauterine growth restriction and Preeclampsia. Update in clinical management.** Hospital Clinic Barcelona. Spain 2015.
3) **Diploma in Reproductive Biotechnology.** Veterinary Faculty, Complutense University of Madrid. Spain 2015.
4) **Reproduction, Breeding and Nutrition in the Rabbit Production.** Polytechnic University of Valencia. Spain 2014.

Clinician experience & externships

July-Sep 2014 **Veterinary Volunteer at Lanta Animal Welfare**. Koh Lanta. Thailand
Main responsibilities included: soft tissues surgeries (reproductive, abdominal, head and neck), wound care and reconstruction, establishment of preventive and internal medicine protocols (dogs, cats, cattle, exotic animals).

2011-2013 **Murube y Barrero, Equine Veterinary Services**. Madrid. Spain
I was involved in the daily ambulatory practice, internal medicine, dental clinic, lameness, reproductive management (Breeding, AI, mare synchronization, foaling and neonatal emergencies).

2009-2012 **Galapavet, Small Animal Ambulatory**. Madrid. Spain
As student, I participated in diagnostics, treatments and surgical support.

2008-2011 **ASAP mascotas**. Madrid. Spain
As student, I participated in the daily taskes of a small ambulatory clinic, veterinarian support and administrative help.